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Title Page

Study Report for Task Order No. UIC-10

MUTAGENICITY TESTING OF WR238605 SUCCINATE

Sponsor: US Army Medical Materiel Development Activity

Contract Number: DAMD17-92-C-2001

Test Article: WR238605 Succinate

Principal Investigator

Barry S. Levine, D.Sc., D.A.B.T.

Study Directors

Patrick T. Curry, Ph.D. (Microbiological Associates, Inc.)
Richard H. C. San, Ph.D. (Microbiological Associates, Inc.)

Completion of Testing Date

(Upon Study Report Submission)

Performing Laboratory

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WR238605 Succinate was tested for point mutations and chromosomal aberrations in three *in vitro* mutagenicity tests. WR238605 Succinate was shown to be negative in the chromosomal aberrations test but was equivocal in the mouse lymphoma assay, which tested for point mutations. Accordingly, a second *in vitro* test for point mutations, the CHO/HGPRT assay, was performed. WR238605 Succinate was negative in this follow-up point mutation assay, and a subsequent confirmatory *in vivo* mutagenicity test is not warranted. Accordingly, the test article does not appear to represent a genotoxic hazard.

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Signature Page

MUTAGENICITY TESTING
OF WR238605 SUCCINATE

Sponsor: US Army Medical Material
Development Activity
Fort Detrick
Frederick, MD 21702-5009

Representative: George J. Schieferstein, Ph.D.

Test Article: WR238605 Succinate

Testing Facility: MICROBIOLOGICAL ASSOCIATES, INC.
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Rockville, MD 20850

as a subcontract to:

TOXICOLOGY RESEARCH LABORATORY (TRL)
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Principal Investigator

5/13/96
Date

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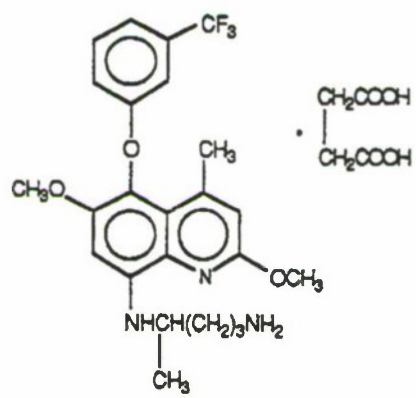
1. SUMMARY

WR238605 Succinate was tested for point mutations and chromosomal aberrations in three *in vitro* mutagenicity tests. WR238605 Succinate was shown to be negative in the chromosomal aberrations test but was equivocal in the mouse lymphoma assay, which tested for point mutations. Accordingly, a second *in vitro* test for point mutations, the CHO/HGPRT assay, was performed. WR238605 Succinate was negative in this follow-up point mutation assay, and a subsequent confirmatory *in vivo* mutagenicity test is not warranted. Accordingly, the test article does not appear to represent a genotoxic hazard.

2. INTRODUCTION

The purpose of this project was to study the *in vitro* mutagenic potential of WR238605 Succinate. The chemical structure of the test article is shown below.



The following *in vitro* assay systems were used:

1. <u>L5178Y TK+/- Mouse Lymphoma Mutagenesis Assay</u>

   This assay tests for specific locus mutations at the thymidine kinase (TK) locus of cultured L5178Y TK+/- mouse lymphoma cells in the presence and absence of rat hepatic microsomal enzymes.

2. <u>Chromosome Aberrations in Chinese Hamster Ovary (CHO) Cells</u>

   This assay evaluates clastogenic potential based upon the ability to induce chromosome aberrations in Chinese hamster ovary (CHO) cells.

3. <u>CHO/HGPRT Mutation Assay</u>

   This assay tests for specific locus mutations at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus of Chinese hamster ovary (CHO) cells.

The mutagenicity studies reported herein were conducted at Microbiological Associates, Inc., and the individual study reports are contained in Appendices 1 - 3. The dosing solutions used in the mutagenicity studies were assayed for test article concentration at UIC after the *in vitro* tests were performed (Appendix

4). They were stored at -65 to -70°C prior to analysis. Since the dosing solutions were added to individual test cultures to result in final test article dose levels, Appendix 5 clarifies which dosing solutions were used for which tested dose levels. Dosing solutions which were added to cultures which were not tested (or tested but not cloned in the mouse lymphoma test) due to excessive toxicity and therefore not assessed for mutagenicity were not analyzed.

3. RESULTS AND DISCUSSION

In the mouse lymphoma mutagenesis assay, the test article was concluded to be negative in the absence of exogenous metabolic activation (Appendix 1). In the presence of exogenous metabolic activation, however, there was a dose-related increase in mutant frequency. Since there was an absolute increase in trifluorothymidine-resistant (TFT) mutant colonies per plate, the dose-related increase in mutant frequency is not attributable to an artificial elevation resulting from increased cytotoxicity (as indicated by reduced total growth). The highest dose level tested, 5.0 µg base/ml, was not included in the evaluation for a positive response because the total growth was below 10%. This leaves only <u>one</u> dose level (2.5 µg base/ml) with 10% or greater total growth exhibiting a mutant frequency two-fold greater than the solvent control. A test article is considered to induce a positive response if a concentration-related increase in mutant frequency is observed and there is <u>more than one dose level</u> with 10% or greater total growth exhibiting a mutant frequency two-fold greater than the solvent control. Therefore, the test article was not evaluated as positive but as equivocal in this study. The size distribution of the mutant colonies for the 2.5 µg base/ml dose level indicated an increase in the frequency of colonies of all sizes when compared to the distribution in the solvent control cultures. This is suggestive of the test article's capability to cause both point mutations (resulting in large mutant colonies) and chromosomal deletions (resulting in small mutant colonies).

Some chemicals (e.g.ethyl methanesulfonate) produce relatively greater numbers of large colonies, while others (e.g. hycanthone) induce predominantly small colony TK-/- mutant colonies. Clive and co-workers (1979) and Hozier *et al.* (1981) have presented evidence to substantiate the hypothesis that the small colony variants carry chromosome aberrations associated with chromosome 11, the chromosome on which the TK locus is located in the mouse (Kozak and Ruddle, 1977). They suggested that large colony mutants received very localized damage, possibly in the form of a point mutation or small deletion within the TK locus, while small colony mutants received damage to collateral loci concordant with the loss of TK activity. This view was recently substantiated by the demonstration of two different classes of mutation in the two alleles of the TK gene at the molecular level (Applegate *et al.*, 1990; Glover *et al.*, 1992). Analysis of the mutant colony size distribution in the study showed a consistent pattern.

In light of the foregoing considerations, even though the test article is not evaluated as positive but as equivocal in the mouse lymphoma assay, the data are indicative of its ability to cause genetic damage. The test article was found to be negative in the *in vitro* cytogenetics assay (chromosome aberrations in CHO cells), both in the presence and in the absence of exogenous metabolic activation (Appendix 2). While the increase in small mutant colonies in the mouse lymphoma assay is suggestive of the test article's capability to cause chromosomal deletions, the negative result in the *in vitro* cytogenetics assay may reflect the differences in the sensitivity of these two mutagenicity tests and the difference in the role of chromosomal deletions in the two assay systems. Accordingly, chromosomal deletions may not be

easily detected in an *in vivo* cytogenetics assay in rats.

Since WR238605 Succinate demonstrated an equivocal response in the mouse lymphoma assay (only in the presence of metabolic activation), a second *in vitro* assay to detect point mutations, e.g. the CHO/HGPRT assay, was performed. The results of this assay were negative in both the presence and absence of rat hepatic microsomal enzymes.

Following completion of the *in vitro* mutagenicity assays, test article concentration was determined in aliquots of dosing solutions which were stored frozen (-65 to 70°C). Almost 90% of the samples (24/27) were within 10% of target concentration. All of the dosing solutions used in study nos. G94BE97.782 (CHO/HGPRT Assay) and G94BE97.330 (Chromosome Aberrations), and the 6 highest concentrations of dosing solutions (out of a total of 9 solutions) used in study no. G94BE97.702 (Mouse Lymphoma Assay) were within target. Since the 6 highest concentrations of dosing solutions used in the Mouse Lymphoma Assay were within target, variations in test article concentration in the 3 lowest dose levels were not considered to have had an impact on the study.

4. CONCLUSION AND RECOMMENDATIONS

Although WR238605 Succinate resulted in a equivocal response in the mouse lymphoma assay (only in the presence of metabolic activation), it was negative in the CHO/HGPRT assay which also detects point mutations. The drug was also negative in the *in vitro* cytogenetics assay. On the basis of these collective results, WR238605 does not appear to represent a potential genotoxic hazard, and further mutagenicity testing is not warranted.

If requested, however, a confirmatory *in vivo* mutagenicity test, such as the Big Blue mutagenesis system in mice, can be performed. The Big Blue mutagenesis system can be used to assess point mutations *in vivo,* and utilizes components of the *lac* operon. The system is ideal in that mutations can be measured in any organ of the animal. Transgenic mice contain a *lacI* target gene carried in a recoverable lambda bacteriophage shuttle vector. The *lacI* gene produces a repressor protein that binds to the *lacZ* operator and blocks synthesis of β-galactosidase by the *lacZ* gene. Mutations in *lacI* can lead to loss of repressor protein activity permitting synthesis of β-galactosidase. The presence of phenotypic alteration of the target *lacI* gene can be detected and easily measured by using a standard calorimetric assay to detect the presence of β-galactosidase.

5. REFERENCES

Applegate, M.L., Moore, M.M., Broder, C.B., Burrel, A., Juhn, G., Kasweck, K.L., Lin, P.F., Wadhams, A., and Hozier, J.C. (1990). Molecular disection of mutations at the heterozygous thymidine kinase locus in mouse lymphoma cells. Proc. Natl. Acad. Sci. USA 87: 51-55.

Clive D, Johnson KO, Spector JFS, Batson AG and Brown MMM (1979). Validation and characterization of the L5178Y TK+/-mouse lymphoma mutagen assay system. *Mutation Res.* 59:61-108.

Glover P, Krehl R, Poorman P and Clive D (1992). Distinctive aspects of mammalian cell mutagenesis. II. Most mutations induced by most mutagens involve several kilobases of DNA. *Environ. Mol. Mutagen.* 19:20.

Hozier J, Sawyer J, Moore M, Howard B, and Clive D (1981). Cytogenetic analysis of L5178Y/$TK^{+/-}$ → $TK^{-/-}$ mouse lymphoma mutagenesis assay system. *Mutation Res.* 84:169-181.

Kozak CA and Ruddle FH (1977). Assignment of the genes for thymidine kinase and galactokinase to *Mus musculus* chromosome 11 and the preferential segregation of this chromosome in Chinese hamster/mouse somatic cell hybrids. *Somatic Cell Genet.* 3:121-133.

Table 1

MUTAGENICITY TESTING OF WR238605 SUCCINATE

Summary of Results

Assay	Doses Tested	Results
Mouse Lymphoma		
-S9	1.0 - 4.0 (μg base/ml)	-
+S9	0.5 - 5.0 (μg base/ml)	equivocal
Chromosomal Aberrations CHO cells		
-S9	1.8 - 3.5 (μg base/ml)	-
+S9	5.6 - 11 (μg base/ml)	-
CHO/HGPRT		
-S9	2.0 - 4.0 (μg base/ml)	-
+S9	6.0 - 10 (μg base/ml)	-

S9 = Metabolic Activation System (rodent liver 9000 x g fraction)

APPENDIX 1

L5178Y/TK$^{+/-}$ MOUSE LYMPHOMA MUTAGENESIS ASSAY REPORT

FINAL REPORT

STUDY TITLE

L5178Y/$TK^{+/-}$ MOUSE LYMPHOMA
MUTAGENESIS ASSAY

TEST ARTICLE

WR238605 Succinate

AUTHORS

Richard H. C. San, Ph.D.
Jane J. Clarke, B.A.

STUDY COMPLETION DATE

January 4, 1996

PERFORMING LABORATORY

MICROBIOLOGICAL ASSOCIATES, INC.
9900 BLACKWELL ROAD
ROCKVILLE, MARYLAND 20850

LABORATORY STUDY NUMBER

G94BE97.702

SPONSOR

Toxicology Research Laboratory
University of Illinois at Chicago
1940 West Taylor Street
Room 312
Chicago, IL 60612-7353

MICROBIOLOGICAL ASSOCIATES, INC.

STATEMENT OF COMPLIANCE

The L5178Y/TK$^{+/-}$ Mouse Lymphoma Mutagenesis Assay, G94BE97.702, was conducted in compliance with the U.S. FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the U.S. EPA GLP Standards 40 CFR 160 and 40 CFR 792, the UK GLP Compliance Programme, the Japanese GLP Standard and the OECD Principles of Good Laboratory Practice in all material aspects with the following exceptions:

> The identity, strength, purity and composition or other characteristics to define the test or control article have not been determined by the testing facility.
>
> The stability of the test or control article under the test conditions has not been determined by the testing facility and is not included in the final report.
>
> Analyses to determine the uniformity, concentration, or stability of the test or control mixtures were not performed by the testing facility.

Richard [signature] 1/4/96

Richard H. C. San, Ph.D. Date
Study Director

QUALITY ASSURANCE STATEMENT

Study Title: L5178Y TK+/- MOUSE LYMPHOMA MUTAGENESIS ASSAY

Study Number: G94BE97.702

Study Director: Richard H. C. San, Ph.D.

This study has been divided into a series of in-process phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment records, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), the UK GLP Compliance Programme, the Japanese GLP Standard, and the OECD Principles of Good Laboratory Practice and to assure that the study is conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 02 SEP 94, TO STUDY DIR 06 SEP 94, TO MGMT 06 SEP 94
PHASE: PROTOCOL REVIEW

INSPECT ON 14 SEP 94, TO STUDY DIR 14 SEP 94, TO MGMT 15 SEP 94
PHASE: DILUTION OF THE TEST ARTICLE

INSPECT ON 06 OCT 94, TO STUDY DIR 06 OCT 94, TO MGMT 28 OCT 94
PHASE: DRAFT REPORT

INSPECT ON 10 JAN 96, TO STUDY DIR 10 JAN 96, TO MGMT 10 JAN 96
PHASE: Draft to Final Report

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Diane B Madsen — 1-10-96

Diane B. Madsen
QUALITY ASSURANCE — DATE

$L5178Y/TK^{+/-}$ MOUSE LYMPHOMA MUTAGENESIS ASSAY

FINAL REPORT

Sponsor: **Toxicology Research Laboratory**
University of Illinois at Chicago
1940 West Taylor Street
Room 312
Chicago, IL 60612-7353

Authorized Representative: **Barry S. Levine, D.Sc., D.A.B.T.**

Performing Laboratory: **MICROBIOLOGICAL ASSOCIATES, INC.**
9900 Blackwell Road
Rockville, Maryland 20850

Test Article I.D.: **WR238605 Succinate**

Test Article Bottle No.: **BM12562**

Test Article Purity: **>99.9% (provided by Sponsor)**

Test Article base mole fraction: **0.8**

Sponsor Project No.: **UIC-10**

MA Study No.: **G94BE97.702**

Test Article Description: **off-white powder**

Storage Conditions: **2-8°C, protected from exposure to light**

Test Article Receipt: **August 26, 1994**

Study Initiation: **September 1, 1994**

Study Director: Richard 1/4/96
Richard H. C. San, Ph.D. Date

Laboratory Manager: Jane J. Clarke 1/4/96
Jane J. Clarke, B.A. Date

MICROBIOLOGICAL ASSOCIATES, INC.

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SUMMARY

Toxicology Research Laboratory's test article WR238605 Succinate was tested in the L5178Y/TK$^{+/-}$ Mouse Lymphoma Mutagenesis Assay in the absence and presence of Aroclor induced rat liver S-9. The non-activated cultures selected for cloning were treated with doses of 4.0 to 1.0 μg base/ml and exhibited total growths from 12% to 101%. The S-9 activated cultures selected for cloning were treated with doses of 5.0 to 0.5 μg base/ml which produced from 6% to 112% total growths.

No non-activated cultures exhibited a mutant frequency which was at least twice the mean mutant frequency of the solvent controls. A dose-dependent response was not noted in the treated cultures. One pair of S-9 activated cultures, with greater than 10% total growth, exhibited mutant frequencies which were at least twice the mean mutant frequency of the solvent controls. A dose-dependent response was noted in the treated cultures. The TFT colonies were sized according to diameter over a range of 0.2 to 1.1 mm. When compared to the solvent control cultures, the treated cultures exhibited an increase in the number of colonies of all sizes. An increase in small colonies is consistent with damage to multiple loci on chromosome 11 in addition to loss of the TK locus.

The results indicate that, under the conditions of this mutagenicity test, test article WR238605 Succinate was negative in the absence of exogenous metabolic activation and equivocal in the presence of exogenous metabolic activation.

INTRODUCTION

Mammalian cell culture systems provide a valuable tool for assessing the genetic hazards of a variety of potentially mutagenic agents. The L5178Y/$TK^{+/-}$ Mouse Lymphoma Mutagenesis Assay was designed to test for specific locus mutations at the thymidine kinase (TK) locus of cultured L5178Y/TK $^{+/-}$ mouse lymphoma cells (Clive and Spector, 1975).

PURPOSE

The purpose of this study was to evaluate the mutagenic potential of the test article or its metabolites using the L5178Y TK/$^{+/-}$ Mouse Lymphoma Mutagenesis Assay.

CHARACTERIZATION OF TEST AND CONTROL ARTICLES

The test article, WR238605 Succinate, was received by Microbiological Associates, Inc. on August 26, 1994 and was assigned the code number 94BE97. The test article was characterized by the Sponsor as a fine, pale yellow powder, which should be stored at 2-8°C in the dark. Its purity was given as >99.9% with a base mole fraction of 0.8.

Upon receipt, the test article was described as a off-white powder and was stored at 2-8°C, protected from exposure to light. At the time of dose administration, the test article was dissolved in dimethylsulfoxide (DMSO) lot 943299, from Fisher Chemical Company. All concentrations presented in this report reflect the correction for base mole fraction. Aliquots of the dosing solutions have been retained by MA. The Sponsor has assumed responsibility for the determination of the stability of the test article.

Ethyl methanesulfonate (EMS), lot A114004, was obtained from Eastman Kodak Chemical Company and was diluted in DMSO to stock concentrations of 50 and 25 µl/ml. 7,12-Dimethylbenz(a)anthracene (7,12-DMBA), lot 9105132310, was obtained from Eastman Kodak Chemical Company and was diluted in DMSO to stock concentrations of 500 and 250 µg/ml. DMSO, lot 943299, was obtained from Fisher Chemical Company.

MATERIALS AND METHODS

Materials

Mammalian Cells:

> L5178Y cells, clone 3.7.2C, were obtained directly from Dr. Donald Clive, Burroughs Wellcome Company, Research Triangle Park, NC. Prior to using L5178Y cells for the mutation assay, the cells were cleansed of spontaneous $TK^{-/-}$ cells by adding a restrictive agent to the culture medium which

selectively kills $TK^{-/-}$ mutants. Cryopreserved L5178Y cells were rapidly thawed and resuspended in flasks at a concentration of $1x10^5$ cells/ml in 100 ml of culture medium. Once normal growth was evident, the cells were cleansed of mutants. THMG (thymidine, hypoxanthine, methotrexate and glycine) was added to each flask at a concentration of 9 μg/ml thymidine, 15 μg/ml hypoxanthine, 0.3 μg/ml methotrexate, and 22.5 μg/ml glycine. The flasks were gassed with 5±1% CO_2 in air and incubated at 37±1°C in an environmental incubator shaker at 125 rpm. After approximately 24 hours, the THMG was removed by pelletizing the cells and decanting the supernatant. The cells were rinsed in 20 ml $F_{10}P$ and reinstated in culture at $3x10^4$ cells/ml in 100 ml of $F_{10}P$ with THG (3 μg/ml thymidine, 5 μg/ml hypoxanthine and 7.5 μg/ml glycine). After approximately 72 hours, the cells were ready to be used in the mutagenesis assay.

Biological Reagents:

Fischer's Medium for Leukemic Cells of Mice with 0.1% Pluronics (F_0P), supplemented with 10% horse serum and 4mM L-glutamine ($F_{10}P$)

Trypsin, 0.1%

Trifluorothymidine (TFT), restrictive agent

Cofactors: 11.25 mg DL-Isocitric acid and 6.0 mg nicotinamide adenine dinucleotide phosphate (NADP), pH 7.0

S-9, 9000 x g supernatant of an Aroclor-1254 induced Sprague-Dawley rat liver homogenate in KCl, lot R504

Cloning medium (C.M.) containing 0.23% Agar

Chemicals:

Solvent for test article and positive controls, dimethylsulfoxide (DMSO), CAS 67-68-5

Ethyl methanesulfonate (EMS), CAS 62-50-0

7,12-Dimethylbenz(a)anthracene (7,12-DMBA), CAS 57-97-6

Methods

The S-9 was prepared according to established procedures. Adult male Sprague-Dawley rats were induced by a single intraperitoneal injection of Aroclor-1254 at a dosage of 500 mg/kg body weight five days prior to sacrifice. The excised tissue was rinsed three times in cold sterile 0.15 M KCl and then homogenized in a Polytron Tissuemizer at a concentration of 1:3 (w/v) in 0.15 M KCl. The supernatant fraction (S-9) was collected

following centrifugation at 9000 x g for 10 minutes at 4±2°C, portioned into aliquots for daily use, and stored frozen at ≤ -70°C until used. Each bulk preparation of S-9 was assayed for sterility and its ability to metabolize 2-aminoanthracene and 7,12-dimethyl-benz(a)anthracene to forms mutagenic to *Salmonella typhimurium* TA100.

Immediately prior to use, the S-9 was mixed with the cofactors and F_0P to contain 250 ul S-9, 6.0 mg NADP, 11.25 mg DL-isocitric acid and 750 μl F_0P/ml S-9 activation mixture and kept on ice until used. The cofactor/F_0P mixture was filter sterilized and adjusted to pH 7.0 prior to the addition of S-9.

The optimal dose levels for the mutagenesis assay were selected following a preliminary toxicity test based on cell population growth relative to the solvent controls. L5178Y cells were exposed to solvent alone and nine concentrations of test article ranging from 5000 to 0.5 μg base/ml for 4 hours in the absence and presence of an exogenous source of metabolic activation. The osmolality of mock cultures containing the solvent only and the top dose was determined. Each tube was gassed with 5 ± 1% CO_2 in air and placed on a Bellco roller drum apparatus rotating at approximately 25 rpm. The final solvent concentration in the culture medium was 1% by volume. The test solutions were prepared under amber lights and kept in darkness during the entire exposure period. After approximately 4 hours, the test article in solution was removed by centrifuging the cells at 1000 rpm for 10 minutes and decanting the supernatant. The cells were washed twice in 10 ml of $F_{10}P$, resuspended in 20 ml $F_{10}P$, gassed with 5 ± 1% CO_2 in air, and replaced on the roller drum apparatus. Washing the cells with $F_{10}P$ instead of F_0P is a deviation from the protocol; however, it had no effect on the integrity of the study. Cell population density was determined 24 and 48 hours after the initial exposure to the test article by removing a sample from each treatment tube, diluting in 0.1% trypsin, incubating at 37±1°C for 10 minutes, and counting the samples with an electronic cell counter. The cultures were adjusted to 3×10^5 cells/ml (if the cell population exceeded 3×10^5 cells/ml) after 24 hours only.

The mutation assay was performed according to a protocol described by Clive and Spector (1975). L5178Y mouse lymphoma cells were cleansed as described previously and resuspended at a cell density of 1×10^6 cells/ml.

Eight concentrations of the test article were added to empty centrifuge tubes. Two control tubes received solvent only and the positive controls were treated with EMS (at final concentrations of 0.5 and 0.25 μl/ml) and 7,12-DMBA (at final concentrations of 5 and 2.5 μg/ml). Four ml of F_0P were added to the non-activated cultures and 4 ml of S-9 reaction mixture were added to the activated cultures. Six ml aliquots of the cell suspension were then dispensed into the tubes to yield 6×10^6 cells/centrifuge tube. The final solvent concentration in the culture medium was 1% by volume. After the treatment period, for 4 hours at 37±1°C, the cells were washed twice with $F_{10}P$ by centrifuging the cultures at 1000 rpm for 10 minutes and decanting the supernatant. The cells were resuspended in $F_{10}P$, gassed with 5±1% CO_2 in air and placed on the roller drum apparatus at 37±1°C.

For expression of the mutant phenotype, the cultures were counted and adjusted to

$3x10^5$ cells/ml (if the cell population exceeded $3x10^5$ cells/ml) at approximately 24 and 48 hours after treatment in 20 and 10 ml total volume, respectively.

For expression of the TK $^{-/-}$ cells, cells were placed in cloning medium (C.M.) containing 0.23% granulated agar. Two flasks per culture to be cloned were labeled with the test article concentration, activation condition, and either TFT or V.C. (viable count). Each flask was prewarmed to 37±1°C, filled with 100 ml C.M., and placed in an incubator shaker at 37±1°C until used. The cells were centrifuged at 1000 rpm for 10 minutes and the supernatant was decanted. The cells were resuspended in 2 ml of cloning medium from the corresponding TFT flask. A $2x10^{-4}$ dilution was carried out by adding 1.0 ml of the TFT flask suspension to a test tube containing 4 ml of $F_{10}P$, adding 1.0 ml of this to 9 ml of $F_{10}P$, and adding 1.0 ml of that dilution to the appropriate V.C. flask containing 100 ml of C.M. After the dilution, 1.0 ml of stock solution of TFT was added to the TFT flask (final concentration of 3 µg/ml) and both this flask and the V.C. flask were placed on the shaker at 125 rpm and 37±1°C. After 15 minutes, the flasks were removed one at a time, and 33 ml of the cell suspension was pipetted into each of three appropriately labeled petri dishes. To accelerate the gelling process, the plates were placed in cold storage (approximately 4°C) for approximately 20 minutes. The plates were removed and incubated at 37±1°C in a humidified 5±1% CO_2 atmosphere for 10-12 days.

Controls

EMS was used as the positive control in the non-activated study at two final concentrations of 0.5 and 0.25 µl/ml. 7,12-DMBA was used as the positive control in the S-9 activated study at two final concentrations of 5.0 and 2.5 µg/ml. The solvent for the test article was used as the solvent control at the same concentration (1% v/v) as that found in the test article-treated groups.

Evaluation of Test Results

Some mathematical calculations were conducted utilizing non-rounded numbers whereas only rounded values were presented. Therefore, reported results of calculations found in the tables may differ slightly from results calculated using only rounded values.

After the incubation period, both the TFT plates and the V.C. plates were scored for the total number of colonies per plate. Three counts per plate were made on an automatic colony counter, and the median count was recorded. The mutation frequency was determined by dividing the average number of colonies in the three TFT plates by the average number of colonies in the three corresponding V.C. plates and multiplying the quotient by the dilution factor (2×10^{-4}). This value is then expressed as the number of mutants per 10^6 clonable cells.

In evaluation of the data, increases in mutant frequencies which occur only at highly toxic concentrations are not considered biologically relevant. All conclusions will be based on sound scientific judgement; however, as a guide to interpretation of the data,

the test article will be considered to induce a positive response if a concentration-related increase in mutant frequency is observed and more than one dose level with 10% or greater total growth exhibits a mutant frequency two-fold greater than the solvent control. A doubling above background at one or more dose levels with 10% or greater total growth with no evidence of a dose-response will be considered equivocal. Test articles not producing a doubling above background at one or more dose levels with 10% or greater total growth will be concluded to be negative.

Criteria for Determination of a Valid Test

The mutant frequency of the positive controls must be at least twice that of the solvent control cultures. The spontaneous mutant frequency of the solvent controls must be between 20 and 100 per 10^6 surviving cells. The cloning efficiency of the solvent controls must be greater than 50%.

Records

All raw data, draft and final reports are maintained in the archives of Microbiological Associates, Inc. located at 9900 Blackwell Road, Rockville, Maryland 20850.

RESULTS AND DISCUSSION

The preliminary toxicity test (Table 1) conducted on test article WR238605 Succinate indicated 100% toxicity down through 5.0 μg base/ml for the non-activated cultures and 100% toxicity down through 50 μg base/ml for the S-9 activated cultures. The osmolality of the solvent control was 458 mOsm/kg and the osmolality of the top dose, 5000 μg base/ml, was 400 mOsm/kg. Based on the results of the initial toxicity test, the doses chosen for the mutagenesis assay ranged from 5.0 to 0.5 μg base/ml for the non-activated and from 10 to 0.125 μg base/ml for the S-9 activated cultures.

The cloning data for test article WR238605 Succinate and positive controls in the absence and presence of an exogenous metabolic activation system are presented in Tables 2 and 4. Total compound toxicity data are presented in Tables 3 and 5. Data for the mutant colony size distributions are presented in Figures 1 and 2.

After a two-day expression period, eleven non-activated cultures and ten S-9 activated cultures were selected for cloning. The non-activated cultures that were cloned had been treated with 4.0, 3.0, 2.5, 2.0, 1.5 and 1.0 μg base/ml which produced a range in suspension growth of 13% to 98%. The S-9 activated cultures that were cloned had been treated with 5.0, 2.5, 1.0, 0.75 and 0.5 μg base/ml which produced a range in suspension growth of 12% to 107%.

No non-activated cultures exhibited a mutant frequency which was at least two times the mean mutant frequency of the solvent controls. The total growths of these cultures ranged from 12% to 101%. A dose-dependent response was not noted in the treated cultures.

One pair of S-9 activated cultures, with ≥10% total growth, exhibited mutant frequencies which were at least two times the mean mutant frequency of the solvent controls. The total growths of the cloned cultures ranged from 6% to 112%. A dose-dependent response was noted in the treated cultures. The TFT colonies for the treated cultures, positive control cultures and solvent control cultures were sized according to diameter over a range from 0.2 to 1.1 mm. The data on colony size distributions showed an increase in the frequency of colonies of all sizes when the treated cultures were compared to the solvent control cultures. An increase in small colonies is consistent with damage to multiple loci on chromosome 11 in addition to loss of the TK locus.

CONCLUSION

The solvent and positive controls fulfilled the requirements for a valid test.

Under the conditions of the assay described in this report, WR238605 Succinate was found to be negative in the absence of exogenous metabolic activation and equivocal in the presence of exogenous metabolic activation. The response of the test article in the presence of S-9 was judged to be equivocal even though there was a dose-response in addition to a doubling of the background mutant frequency a one dose level.

REFERENCES

Clive, D. and J.F.S. Spector. 1975. Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells. Mutation Research 31:17-29.

TABLE 1

PRELIMINARY COMPOUND TOXICITY TEST USING WR238605 Succinate

Test Article Concentration (µg base/ml)	Cell Concentration ($X 10^6$) Day 1	Day 2	Suspension Growth[a] Total[b]	% of Control[c]
WITHOUT ACTIVATION				
5000	0.147	0.023	0.0	0
1000	0.012	0.005	0.0	0
500	0.034	0.006	0.0	0
100	0.111	0.031	0.0	0
50	0.025	0.011	0.0	0
10	0.023	0.011	0.0	0
5.0	0.047	0.019	0.0	0
1.0	1.408	1.470	23.0	92
0.5	1.412	1.409	22.1	88
Solvent 1	1.423	1.518	24.0	
Solvent 2	1.517	1.545	26.0	
WITH S-9 ACTIVATION				
5000	0.218	0.171	0.0	0
1000	0.182	0.025	0.0	0
500	0.068	0.012	0.0	0
100	0.067	0.019	0.0	0
50	0.027	0.008	0.0	0
10	0.395	0.232	1.0	6
5.0	0.416	0.309	1.4	9
1.0	0.601	0.899	6.0	37
0.5	0.835	1.391	12.9	79
Solvent 1	1.010	1.450	16.3	
Solvent 2	1.022	1.443	16.4	

[a] - Cultures containing <$0.3x10^6$ cells/ml on day 1 and 2 are considered as having 0% total suspension growth.

[b] - Total suspension growth = (Day 1 cell conc. / $0.3x10^6$ cells/ml) x (Day 2 cell conc. / Day 1 adjusted cell conc.)

[c] - % of control suspension growth = (total treatment suspension growth / average solvent control total suspension growth) x 100

TABLE 2

CLONING DATA FOR L5178Y/TK$^{+/-}$ MOUSE LYMPHOMA CELLS
TREATED WITH WR238605 Succinate
IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

Test Article Concentration (µg base/ml)	Ave #/ TFT Plate[a]	TFT Stand Dev	Ave #/ V.C. Plate[a]	V.C. Stand Dev	Mutant Frequency[b]	Induced Mutant Frequency[c]	% Total Growth[d]
4.0 A	18/3	4	137/3	4	26	0	12
4.0 B	++		++				
3.0 A	19/3	1	134/3	13	28	2	34
3.0 B	25/3	2	140/3	3	36	10	30
2.5 A	15/3	2	164/3	3	18	-8	59
2.5 B	18/3	1	122/3	2	30	4	53
2.0 A	19/3	2	148/3	5	26	0	77
2.0 B	13/3	3	159/1	0	16	-10	77
1.5 A	20/3	3	139/3	3	29	3	83
1.5 B	16/3	1	128/3	7	25	-1	77
1.0 A	17/3	4	151/3	5	23	-3	101
1.0 B	20/3	3	152/3	22	26	0	99
Solvent 1	17/2	1	174/3	7	20		
Solvent 2	18/3	1	118/3	6	31		
Mean Solvent Mutant Frequency= 26							
Positive Control - Ethyl Methanesulfonate (µl/ml)							
0.50	374/3	26	92/3	15	813	791	34
0.25	287/3	7	151/3	2	380	358	71
Solvent 1	20/3	1	167/3	9	24		
Solvent 2	15/3	2	161/3	9	19		
Mean Solvent Mutant Frequency= 22							

++ - Too Toxic To Clone

[a] - Average # of colonies per plate and # of plates scored

[b] - Mutant frequency (per 10^6 surviving cells) = [(Average # TFT colonies / average # VC colonies) x $2x10^{-4}$] x 10^6

[c] - Induced mutant frequency (per 10^6 surviving cells) = mutant frequency - average mutant frequency of solvent controls

[d] - % total growth = (% suspension growth x % cloning growth) / 100

TABLE 3

TOTAL COMPOUND TOXICITY DATA FOR L5178Y/TK$^{+/-}$ MOUSE LYMPHOMA CELLS TREATED WITH WR238605 Succinate IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

Test Article Concentration (µg base/ml)	Cell Concentration (X 10^6) Day 1	Day 2	Suspension Growth[a] Total[b]	%Cntl[c]	Cloning Growth Ave VC	%Cntl[d]	%Total Growth[e]
5.0 A	0.030	0.098	0.0	0	++		
5.0 B	0.049	0.156	0.0	0	++		
4.0 A	0.220	0.854	2.8	13	137	94	12
4.0 B	0.102	0.330	1.1	5	++		
3.0 A	0.619	1.139	7.8	37	134	92	34
3.0 B	0.522	1.135	6.6	31	140	96	30
2.5 A	0.788	1.264	11.1	53	164	112	59
2.5 B	0.913	1.300	13.2	63	122	84	53
2.0 A	0.994	1.453	16.0	76	148	101	77
2.0 B	0.985	1.365	14.9	71	159	109	77
1.5 A	1.114	1.473	18.2	87	139	95	83
1.5 B	1.130	1.462	18.4	88	128	88	77
1.0 A	1.167	1.590	20.6	98	151	103	101
1.0 B	1.161	1.554	20.0	95	152	104	99
Solvent 1	1.209	1.620	21.8		174		
Solvent 2	1.171	1.551	20.2		118		
Positive Control - Ethyl Methanesulfonate (µl/ml)							
0.50	0.854	1.282	12.2	60	92	56	34
0.25	1.019	1.383	15.7	77	151	92	71
Solvent 1	1.257	1.522	21.3		167		
Solvent 2	1.131	1.568	19.7		161		

++ - Too Toxic To Clone

[a] - Cultures containing <0.3x10^6 cells/ml on day 1 and 2 are considered as having 0% total suspension growth.

[b] - Total suspension growth = (Day 1 cell conc. / 0.3x10^6 cells/ml) x (Day 2 cell conc. / Day 1 adjusted cell conc.)

[c] - % of control suspension growth = (total treatment suspension growth / average solvent control total suspension growth) x 100

[d] - % control cloning growth = (average V.C. of treated culture / average V.C. of solvent control) x 100

[e] - % total growth = (% suspension growth x % cloning growth) / 100

TABLE 4

**CLONING DATA FOR L5178Y/TK+/- MOUSE LYMPHOMA CELLS
TREATED WITH WR238605 Succinate
IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION**

Test Article Concentration (µg base/ml)	Ave #/ TFT Plate[a]	TFT Stand Dev	Ave #/ V.C. Plate[a]	V.C. Stand Dev	Mutant Frequency[b]	Induced Mutant Frequency[c]	% Total Growth[d]
5.0 A	47/3	2	64/3	3	147	110	6
5.0 B	35/3	4	60/3	7	117	80	6
2.5 A	55/3	7	100/3	2	110	73	17
2.5 B	48/3	7	91/3	7	105	68	14
1.0 A	38/3	7	119/3	1	64	27	81
1.0 B	39/3	13	137/3	8	57	20	102
0.75 A	40/3	4	125/3	16	64	27	93
0.75 B	38/3	11	115/3	8	66	29	83
0.50 A	27/3	1	131/2	5	41	4	106
0.50 B	23/3	1	147/3	3	31	-6	112
Solvent 1	23/2	5	119/3	14	39		
Solvent 2	25/3	4	146/3	6	34		
Mean Solvent Mutant Frequency= 37							
Positive Control - 7,12 Dimethylbenz(a)anthracene (µg/ml)							
5.0	122/3	3	32/3	6	763	732	7
2.5	119/3	10	97/3	10	245	214	53
Solvent 1	18/3	3	122/3	4	30		
Solvent 2	23/3	2	144/3	5	32		
Mean Solvent Mutant Frequency= 31							

[a] - Average # of colonies per plate and # of plates scored

[b] - Mutant frequency (per 10^6 surviving cells) = [(Average # TFT colonies / average # VC colonies) x $2x10^{-4}$] x 10^6

[c] - Induced mutant frequency (per 10^6 surviving cells) = mutant frequency - average mutant frequency of solvent controls

[d] - % total growth = (% suspension growth x % cloning growth) / 100

TABLE 5

TOTAL COMPOUND TOXICITY DATA FOR L5178Y/TK$^{+/-}$ MOUSE LYMPHOMA CELLS TREATED WITH WR238605 Succinate IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

Test Article Concentration (µg base/ml)	Cell Concentration (X 10⁶) Day 1	Day 2	Suspension Growth[a] Total[b]	%Cntl[c]	Cloning Growth Ave VC	%Cntl[d]	%Total Growth[e]
10.0 A	0.364	0.345	1.4	9	++		
10.0 B	0.311	0.288	1.0	7	++		
5.0 A	0.370	0.443	1.8	12	64	48	6
5.0 B	0.380	0.450	1.9	13	60	45	6
2.5 A	0.429	0.683	3.3	22	100	75	17
2.5 B	0.411	0.676	3.1	21	91	69	14
1.0 A	0.788	1.531	13.4	90	119	90	81
1.0 B	0.875	1.514	14.7	99	137	103	102
0.75 A	0.818	1.625	14.8	99	125	94	93
0.75 B	0.825	1.544	14.2	95	115	87	83
0.50 A	0.879	1.624	15.9	107	131	99	106
0.50 B	0.880	1.542	15.1	101	147	111	112
Solvent 1	0.832	1.624	15.0		119		
Solvent 2	0.846	1.561	14.7		146		
Positive Control - 7,12 Dimethylbenz(a)anthracene (µg/ml)							
5.0	0.494	0.864	4.7	30	32	24	7
2.5	0.731	1.365	11.1	72	97	73	53
Solvent 1	0.912	1.531	15.5		122		
Solvent 2	0.959	1.459	15.5		144		

++ - Too Toxic To Clone

a - Cultures containing <0.3x10⁶ cells/ml on day 1 and 2 are considered as having 0% total suspension growth.

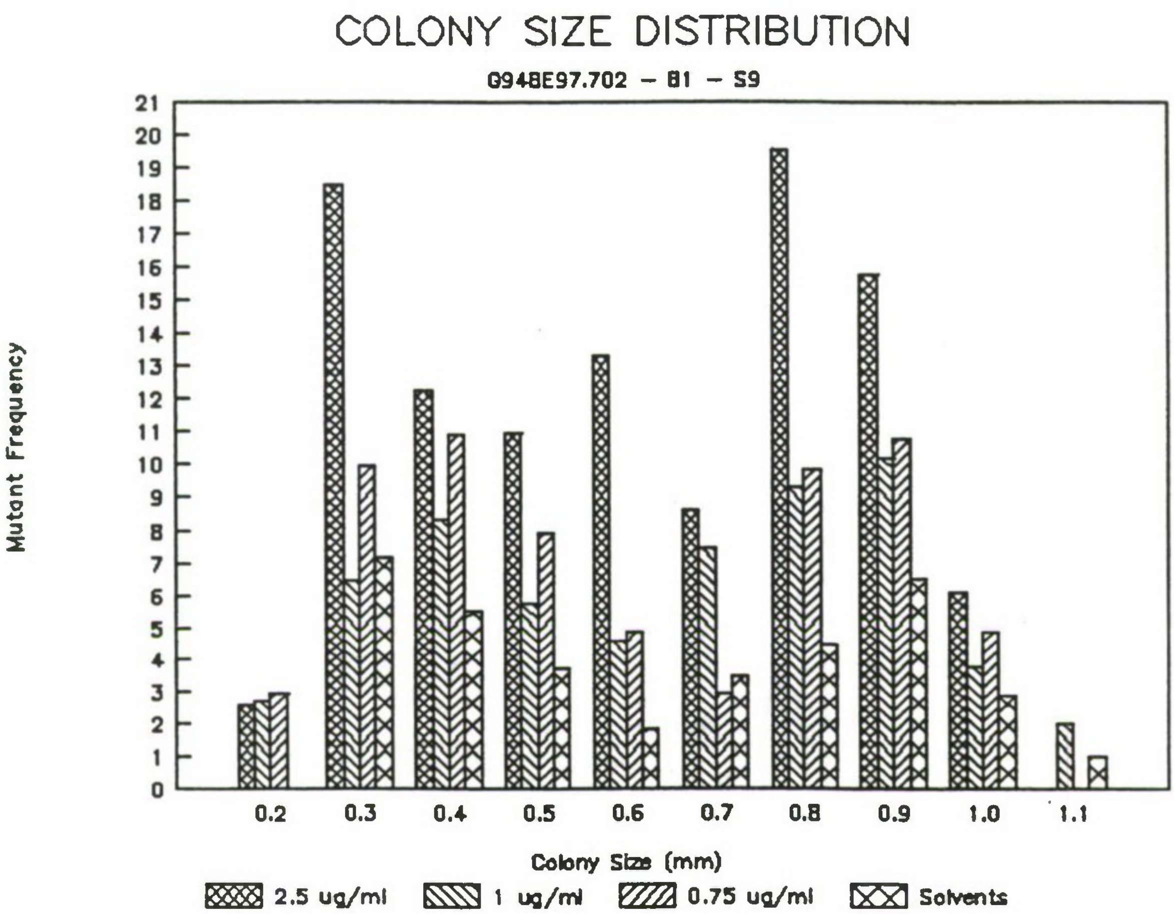
b - Total suspension growth = (Day 1 cell conc. / 0.3x10⁶ cells/ml) x (Day 2 cell conc. / Day 1 adjusted cell conc.)

c - % of control suspension growth = (total treatment suspension growth / average solvent control total suspension growth) x 100

d - % control cloning growth = (average V.C. of treated culture / average V.C. of solvent control) x 100

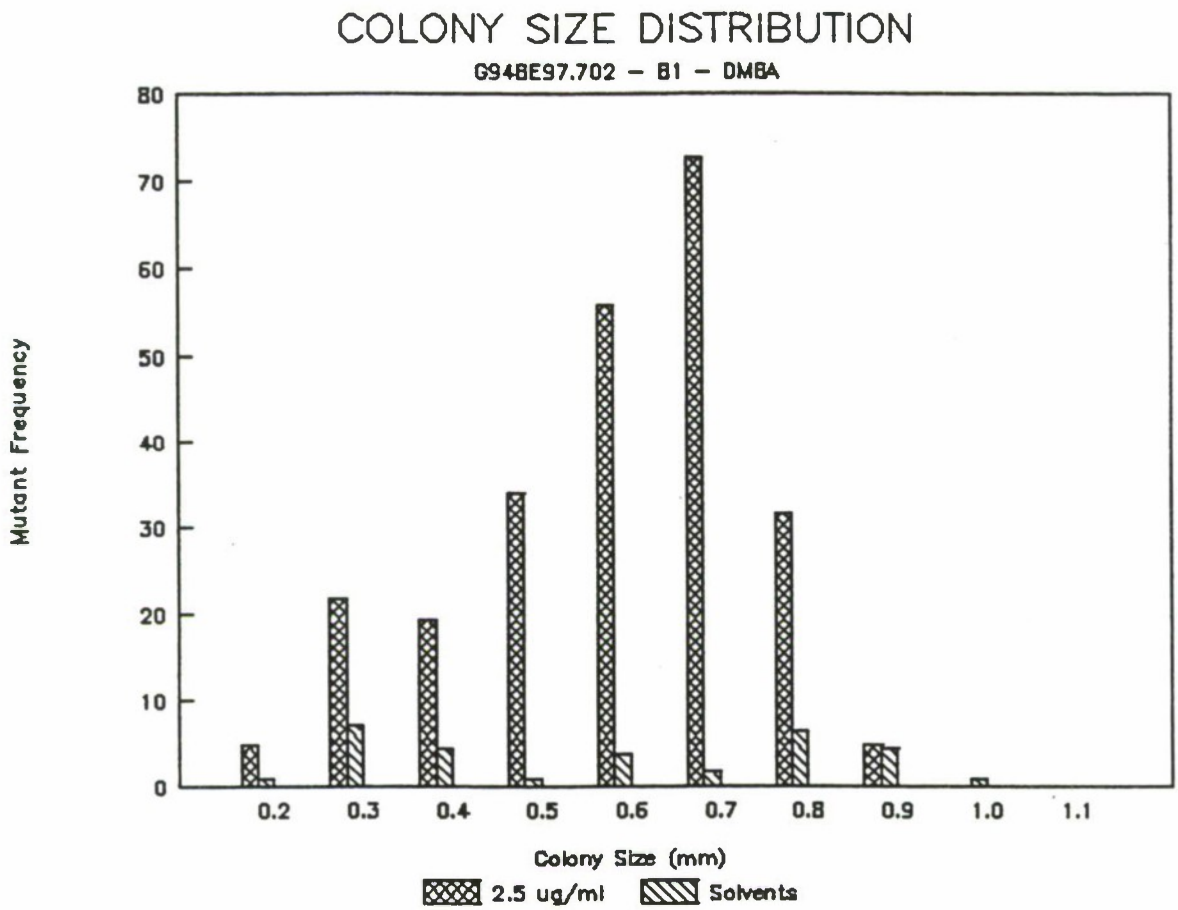
e - % total growth = (% suspension growth x % cloning growth) / 100

FIGURE 1



COLONY SIZE DISTRIBUTION
G94BE97.702 – B1 – S9
Mutant Frequency
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
Colony Size (mm)
2.5 ug/ml
1 ug/ml
0.75 ug/ml
Solvents

FIGURE 2



COLONY SIZE DISTRIBUTION
G94BE97.702 – B1 – DMBA
80
70
60
50
40
30
20
10
0
Mutant Frequency
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
Colony Size (mm)
2.5 ug/ml
Solvents

APPENDIX I

Mouse Lymphoma Historical Control Data
1991-1993

	Nonactivated			S-9 Activated		
	Sol	0.5μl/ml EMS	0.25μl/ml EMS	Sol	5.0μg/ml DMBA	2.5μg/ml DMBA
Mean MF	39.1	848.6	447.3	46.9	592.1	236.4
SD	12.3	173.8	73.5	12.6	216.2	61.6
Maximum	80.0	1314.0	691.0	87.0	1358.0	517.0
Minimum	22.0	458.0	294.0	22.0	167.0	100.0

Sol = Solvent control (Fischer's medium, distilled water, saline, DMSO, ethanol, acetone or vehicle supplied by Sponsor)
EMS = Ethylmethanesulfonate
DMBA = Dimethylbenz(a)anthracene
MF = Mutant frequency per 10^6 clonable cells
SD = Standard deviation

APPENDIX II

Study Protocol

PROTOCOL AMENDMENT 1

SPONSOR: **Toxicology Research Laboratory**

TEST ARTICLE I.D.: **WR238605 Succinate**

MA STUDY NO: **G94BE97.702**

SPONSOR PROJECT NO.: **UIC-10**

PROTOCOL TITLE: **L5178Y/TK$^{+/-}$ Mouse Lymphoma Mutagenesis Assay**

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1. **LOCATION:** Page 1, § 3.1;

   AMENDMENT: Change storage temperature to 2-6°C.

   REASON FOR THE AMENDMENT: To be consistent with the Standard Operating Procedures of the testing facility.

APPROVALS:

STUDY DIRECTOR	9/6/94 DATE
SPONSOR REPRESENTATIVE	9/15/94 DATE

MICROBIOLOGICAL ASSOCIATES, INC.

APPROVED

QC 9-6-94

Received by RA/QA 9-1-94

MA Study Number: G94BE97.702

L5178Y/TK$^{+/-}$ MOUSE LYMPHOMA MUTAGENESIS ASSAY

1.0 PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test article based on quantitation of forward mutations at the thymidine kinase locus of L5178Y mouse lymphoma cells.

2.0 SPONSOR

2.1	Name:	**Toxicology Research Laboratory**
2.2	Address:	University of Illinois at Chicago Department of Pharmacology 1940 West Taylor Street Chicago, IL 60612-7353
2.3	Representative:	Barry S. Levine, D.Sc., D.A.B.T.
2.4	Sponsor Project #:	UIC-10. This study will be conducted by Microbiological Associates,Inc. and is being sponsored by the Toxicology Research Laboratory, UIC under a contract from U.S. Army Medical Research Acquisition Activity (No. DAMD 17-92-C-2001). Dr. George Schieferstein, Contracting Officer's Representative.

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1 Test Article: **WR238605 Succinate**

Storage: 0 - 4°C
Ambient humidity conditions
Protect from light

3.2 Controls:

Negative:	Test article solvent	
Positive:	Ethyl methanesulfonate (EMS) 7,12-dimethylbenz(a)anthracene (DMBA)	

3.3 Determination of Strength, Purity, etc.

The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article and the stability and strength of the dosing solutions.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1 Name: Genetic and Cellular Toxicology Division
Microbiological Associates, Inc.

4.2 Address: 9900 Blackwell Road
Rockville, MD 20850

4.3 Study Director: Richard H. C. San, Ph.D.

5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date: 9/13/94

5.2 Proposed Experimental Completion Date: 10/17/94

5.3 Proposed Report Date: 10/28/94

6.0 TEST SYSTEM

L5178Y/TK$^{+/-}$ mouse lymphoma cells are heterozygous at the normally diploid thymidine kinase (TK) locus. L5178Y/TK$^{+/-}$, clone 3.7.2C, were received from Dr. Donald Clive, Burroughs Wellcome Company, Research Triangle Park, North Carolina. Each freeze lot of cells has been tested and found to be free of mycoplasma contamination. This system has been demonstrated to be sensitive to the mutagenic activity of a variety of chemicals.

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The mammalian mutation assay will be performed by exposing duplicate cultures of L5178Y/TK$^{+/-}$ cells to a minimum of eight concentrations of test article as well as positive and negative (solvent) controls. Exposures will be for 4 hours in the presence and absence of an S-9 activation system. Following a two day expression period, with daily cell population adjustments, cultures demonstrating 0% to 90% growth inhibition will be cloned, in triplicate, in restrictive medium containing soft agar to select for the mutant phenotype. After a 10 to 12 day selection period, mutant colonies will be enumerated. The mutagenic potential of the test article will be measured by its ability to induce TK$^{+/-}$ $\rightarrow$ TK$^{-/-}$ mutations. For those test articles demonstrating a positive response, mutant colonies will be sized as a indication of mechanism of action.

7.1 Selection of solvent

Unless the Sponsor has indicated the test article solvent, a solubility determination will be conducted to measure the maximum soluble concentration in a variety of solvents. Solvents compatible with this test system, in order of preference, include, but are not limited to, culture medium or distilled water (CAS 7732-18-5), dimethylsulfoxide (CAS 67-68-5), ethanol

(CAS 64-17-5) and acetone CAS 67-64-1). The solvent of choice will be that solvent, selected in order of preference, that permits preparation of the highest soluble stock concentration, up to a maximum of 500 mg/ml.

7.2 Dose Selection

In the preliminary toxicity test, L5178Y/$TK^{+/-}$ cells will be exposed to solvent alone and to at least nine concentrations of test article, the highest concentration being the lowest insoluble dose in treatment medium but not to exceed 5000 μg/ml. The pH of the treatment medium will be adjusted, if necessary, to maintain a neutral pH in the treatment medium. The osmolality of the highest soluble treatment condition will also be measured. After a 4-hour treatment in the presence and absence of S-9 activation, cells will be washed free of chemical and cultured in suspension for two days post-treatment, with cell concentration adjustment on the first day.

Selection of dose levels for the mutation assay will be based on reduction of suspension growth after treatment in the preliminary toxicity test. Unless specified otherwise by the Sponsor, the high dose for the mutation assay will be that concentration exhibiting approximately 100% growth inhibition. The low dose will be selected to exhibit 0% growth inhibition. In the event that the test article cannot be dissolved in solvent or in treatment medium at a high enough concentration to be toxic, the Sponsor will be consulted prior to dose selection.

7.3 Route and Frequency of Administration

Cell cultures will be treated for 4 hours by way of a vehicle compatible with the system, both in the presence and absence of metabolic activation. This technique of administration has been demonstrated to be effective in the detection of chemical mutagens in this system.

7.4 Exogenous Metabolic Activation

Aroclor 1254-induced rat liver S-9 will be used as the metabolic activation system. The source of S-9 will be adult male Sprague-Dawley rats induced by a single injection of Aroclor 1254 at a dose level of 500 mg/kg body weight five days prior to sacrifice. The S-9 will be batch prepared and stored frozen approximately -70°C until used.

Immediately prior to use, the S-9 will be thawed and mixed with a cofactor pool to contain 11.25 mg DL-isocitric acid, 6 mg NADP, and 0.25 ml S-9 homogenate per ml in Fischer's Media for Leukemic Cells of Mice with 0.1% Pluronics (F_0P). The S-9 mix will be adjusted to pH 7.

7.5 Controls

7.5.1 Solvent Control

The solvent for the test article will be used as the solvent control.

7.5.2 Positive Controls

Ethyl methanesulfonate (EMS) will be used at two concentrations within the range of 0.2 to 0.6 μl/ml as the positive control for the nonactivated test system. For the S-9 activated system, 7,12-dimethylbenz(a)anthracene (DMBA) will be used at two concentrations within the range of 2 to 6 μg/ml.

7.6 Preparation of Target Cells

Prior to use in the assay, L5178Y/$TK^{+/-}$ cells will be cleansed to reduce the frequency of spontaneously occurring $TK^{-/-}$ cells. Using the procedure described by Clive (1975), L5178Y cells will be cultured for 24 hours in the presence of thymidine, hypoxanthine, methotrexate and glycine to poison the $TK^{-/-}$ cells.

L5178Y/$TK^{+/-}$ cells will be prepared at 1 x 10^6 cells/ml in 50% conditioned $F_{10}P$ and 50% F_0P. If cultures are to be treated with more than 100 μl of test article dosing solution, the cell concentration may be adjusted.

7.7 Identification of the Test System

The test system will be identified by the study number, the treatment condition and test phase.

7.8 Treatment of Target Cells

Treatment will be carried out in conical tubes by combining 6 x 10^6 L5178Y/$TK^{+/-}$ cells and 100 μl dosing solution of test or control article in solvent or solvent alone in a total volume of 10 ml F_0P medium or S-9 activation mixture. A total of eight concentrations of test article will be tested in duplicate. All pH adjustments will be performed prior to adding S-9 or target cells to the treatment medium. Volumes of test article dosing solution in excess of 100 μl may be used if required to achieve the target final concentration in treatment medium. Treatment tubes will be gassed with 5 $\pm$ 1% $C0_2$ in air, capped tightly, and incubated with mechanical mixing for 4 hours at 37 $\pm$ 1°C. The preparation and addition of the test article dosing solutions will be carried out under amber lighting and the cells will be incubated in the dark during the 4-hour exposure period.

7.9 Expression of the Mutant Phenotype

At the end of the exposure period, the cells will be washed twice with F_0P and collected by centrifugation. The cells will be resuspended in 20 ml $F_{10}P$, gassed with 5 ± 1% CO_2 in air and cultured in suspension at 37 ± 1°C for two days following treatment. Cell population adjustments to 0.3 x 10^6 cells/ml will be made at 24 and 48 hours.

7.10 Selection of the Mutant Phenotype

For selection of the trifluorothymidine (TFT)-resistant phenotype, cells from up to ten treatment conditions demonstrating from 0% to 90% suspension growth inhibition will be plated into three replicate dishes at a density of 1 x 10^4 cells/100mm dish in cloning medium ($F_{10}P$ with 0.23% agar) containing 2-4 μg TFT/ml. For estimation of cloning efficiency at the time of selection, 200 cells/100mm dish will be plated in triplicate in cloning medium free of TFT (viable cell (VC) plates). Plates will be incubated at 37 ± 1°C in a humidified atmosphere of 5 ± 1% CO_2 for 10-12 days.

The total number of colonies per plate will be determined for both the TFT-plates and the VC plates. Three counts per plate will be made using an automatic colony counter, and the median count will be recorded. If the automatic counter cannot be used, the colonies will be counted manually. In the event the test article demonstrates a significant induction in mutant frequency as compared to the solvent controls, the diameters of the TFT colonies will be determined over a range of 0.2 to 1.1 mm.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

8.1 Negative Controls

The spontaneous mutant frequency of the solvent control cultures must be within 20 to 100 TFT-resistant mutants per 10^6 surviving cells. The cloning efficiency of the solvent control group must be greater than 50%.

8.2 Positive Controls

The mutant frequency of the positive controls must be at least twice that of the solvent control.

9.0 EVALUATION OF TEST RESULTS

The cytotoxic effects of each treatment condition are expressed relative to the solvent-treated control for suspension growth over two days post-treatment and for total growth (suspension growth corrected for plating efficiency at the time of selection). The mutant frequency for each treatment condition is calculated by dividing the mean number of colonies on the TFT-plates by the mean number of colonies on the VC-plates and multiplying by the dilution factor (2 x 10^{-4}), and is

expressed as TFT-resistant mutants per 10^6 surviving cells.

In evaluation of the data, increases in mutant frequencies which occur only at highly toxic concentrations (ie, less than 10% total growth) are not considered biologically relevant. All conclusions will be based on sound scientific judgement; however, as a guide to interpretation of the data, the test article will be considered to induce a positive response if a concentration-related increase in mutant frequency is observed and more than one dose level with 10% or greater total growth exhibiting a mutant frequency two-fold greater than the solvent control. A doubling above background at one or more dose levels with 10% or greater total growth with no evidence of a dose-response will be considered equivocal. Test articles not producing a doubling above background at one or more dose levels with 10% or greater total growth will be concluded to be negative.

10.0 REPORT

A report of the results of this study will be prepared by the Testing Laboratory and will accurately describe all methods used in the generation and analysis of data.

Results presented will include, but not be limited to:

- cell type used, number of cultures, methods for maintenance of cell cultures
- rationale for selection of concentrations and number of cultures
- test conditions: composition of media, CO_2 concentration, concentration of test substance, vehicle, incubation temperature, incubation time, duration of treatment, cell density during treatment, type of metabolic activation system, positive and negative controls, length of expression period, selective agent
- method used to enumerate numbers of viable and mutant cells
- dose-response relationship, if applicable
- distribution of the mutant colony diameter for each significantly elevated dose level of test article, solvent and positive control (only when the test article induces a positive response)
- positive and solvent control historical data

11.0 RECORDS AND ARCHIVES

Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates, Inc. in accordance with the relevant Good Laboratory Practice Regulations.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol was written according to the OECD Guideline 476 (Genetic Toxicology: *In Vitro* Mammalian Cell Gene Mutation Tests), April, 1984; and the EPA Health Effects Testing Guidelines, Subpart 798.5300 (Detection of Gene Mutations in Somatic Cells in Culture) Fed. Register, vol. 50, September, 1985 with revisions Fed. Register, vol. 52, May, 1987. A confirmatory assay will be required for full OECD and EPA guideline compliance.

This study will be performed in compliance with the provisions of the Good Laboratory Practice Regulations for Nonclinical Laboratory Studies.

Will this study be submitted to a regulatory agency? Yes

If so, to which agency or agencies? US FDA

Unless arrangements are made to the contrary, the Test Article will be returned to the Sponsor at the conclusion of the study. In addition, does the Sponsor request that samples of the Test Article dosing solutions be returned? Yes

13.0 REFERENCES

Clive, D. and Spector, J.F.S. Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells. Mutation Res. 31:17-29, 1975.

14.0 APPROVAL

Barry S. Levine 6/29/94

Barry S. Levine, D.Sc., D.A.B.T. (Sponsor Representative) Date

G Schieferstein 7/5/94

Dr. George Schieferstein (US Army Contracting Officer's Representative) Date

Rick [illegible] 9/1/94

MA Study Director Date

APPENDIX 2

CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY (CHO) CELLS REPORT

FINAL REPORT

Study Title

CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY (CHO) CELLS

Test Article

WR238605 Succinate

Authors

Patrick T. Curry, Ph.D.
Elizabeth Schadly, B.S.

Study Completion Date

January 2, 1996

Performing Laboratory

Microbiological Associates, Inc.
9900 Blackwell Road
Rockville, Maryland 20850

Laboratory Study Number

G94BE97.330

Sponsor

Toxicology Research Laboratory
University of Illinois at Chicago
1940 West Taylor Street
Room 312
Chicago, IL 60612-7353

MICROBIOLOGICAL ASSOCIATES, INC.

STATEMENT OF COMPLIANCE

The cytogenetics study, Chromosome Aberrations in Chinese Hamster Ovary (CHO) Cells, G94BE97.330, was conducted in compliance with the US FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the US EPA GLP Standards 40 CFR 160 and 40 CFR 792, the UK GLP Compliance Programme, the Japanese GLP Standard and the OECD Principles of Good Laboratory Practice in all material aspects with the following exceptions:

> The identity, strength, purity and composition or other characteristics to define the test or control article have not been determined by the testing facility.
>
> The stability of the test or control article under the test conditions has not been determined by the testing facility and is not included in the final report.
>
> Analyses to determine the uniformity, concentration, or stability of the test or control mixtures were not performed by the testing facility.

Patrick T. Curry

Patrick T. Curry, Ph.D.
Study Director

1-2-96
Date

QUALITY ASSURANCE STATEMENT

Study Title: CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY (CHO) CELLS

Study Number: G94BE97.330

Study Director: Patrick T. Curry, Ph.D.

This study has been divided into a series of in-process phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment records, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), the UK GLP Compliance Programme, the Japanese GLP Standard, and the OECD Principles of Good Laboratory Practice and to assure that the study is conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 02 SEP 94, TO STUDY DIR 02 SEP 94, TO MGMT 02 SEP 94
PHASE: PROTOCOL REVIEW

INSPECT ON 06 OCT 94, TO STUDY DIR 07 OCT 94, TO MGMT 07 OCT 94
PHASE: DILUTION OF THE TEST ARTICLE AND POSITIVE CONTROLS

INSPECT ON 02 NOV 94, TO STUDY DIR 02 NOV 94, TO MGMT 04 NOV 94
PHASE: DRAFT REPORT

INSPECT ON 04 JAN 96, TO STUDY DIR 04 JAN 96, TO MGMT 05 JAN 96
PHASE: Draft to Final Report

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Claire L. Courtemanche ____________________ Jan 5, 1996

Claire L. Courtemanche
QUALITY ASSURANCE

DATE

CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY (CHO) CELLS

FINAL REPORT

Sponsor: **Toxicology Research Laboratory**
University of Illinois at Chicago
1940 West Taylor Street
Room 312
Chicago, IL 60612-7353

Authorized Representative: **Barry S. Levine, D.Sc., D.A.B.T.**

Performing Laboratory: **Microbiological Associates, Inc. (MA)**
9900 Blackwell Road
Rockville, Maryland 20850

Test Article I.D.: **WR238605 Succinate**

Purity: **>99.9%, provided by Sponsor**

Base Mole Fraction: **0.8, provided by Sponsor**

MA Study No.: **G94BE97.330**

Test Article Description: **off-white powder**

Storage Conditions: **2-8°C, protected from light**

Test Article Receipt: **August 26, 1994**

Study Initiation: **August 31, 1994**

Study Director: Patrick T. Curry — 1-2-96
Patrick T. Curry. Ph.D. — Date

Associate Study Director: Donald L. Putman — 1-2-96
Donald L. Putman, Ph.D. — Date

Laboratory Supervisor: Elizabeth H. Schadly — 1-2-96
Elizabeth H. Schadly, B.S. — Date

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SUMMARY

The test article, WR238605 Succinate, was tested in the chromosome aberration assay in the absence and presence of an Aroclor-induced S-9 activation system using Chinese hamster ovary (CHO) cells. The assay was performed in two phases. The first phase, a preliminary toxicity test, was performed to establish a dose range and cell collection times for the chromosome aberration assay. The second phase, the chromosome aberration assay, was used to evaluate the clastogenic potential of the test article.

Dimethylsulfoxide (DMSO) was determined to be the solvent of choice based on the solubility of the test article in the solvent and compatibility of the solvent with the target cells. The test article was soluble in DMSO at a maximum concentration of approximately 357 mg base/ml. The limit of solubility of the test article in test medium was 224 μg base/ml.

The highest dose level tested in the preliminary toxicity test, 224 μg base/mL, was selected because it was the highest soluble concentration in treatment medium. In the preliminary cytotoxicity test, toxicity (measured by mitotic inhibition relative to the solvent control) was 26% in the nonactivated test system at the 2.24 μg base/ml dose level. There was also an increase in the average generation time (AGT) from approximately 12 hours to approximately 13 hours. In the nonactivated test system, complete mitotic inhibition was seen at dose levels of 6.72 μg base/ml and greater. In the S-9 activated test system toxicity (mitotic inhibition) was 47% at the 6.72 μg base/ml dose level. The AGT was increased from approximately 12 hours to approximately 21 hours. Complete mitotic inhibition was seen at dose levels of 22.4 μg base/ml and greater in the S-9 activated system. The highest dose levels selected for testing in the chromosome aberration assay were 5.4 and 18 μg base/ml for the nonactivated and S-9 activated test systems, respectively. Due to the cell cycle delay (increased AGT) observed in the preliminary toxicity test, the cell harvest times were set at 18 and 20 hours after treatment initiation for the nonactivated and S-9 activated test systems, respectively.

In the chromosome aberration assay, toxicity (mitotic inhibition) was approximately 69% at the highest dose level analyzed in the nonactivated study (3.5 μg base/ml); and approximately 72% at the highest dose level analyzed in the S-9 activated study (11 μg base/ml). Excessive toxicity precluded analysis of dose levels 4.3 and 5.4 μg base/ml in the nonactivated assay, and 14 and 18 μg base/ml in the S-9 activated assay. No significant increases in chromosome aberrations were observed in the nonactivated or S-9 activated test systems relative to the solvent control group. Based on the findings of this study, WR238605 Succinate was concluded to be negative in the chromosome aberration assay using Chinese hamster ovary (CHO) cells.

PURPOSE

The purpose of this study was to evaluate the clastogenic potential of a test article based upon its ability to induce chromosome aberrations in Chinese hamster ovary (CHO) cells.

CHARACTERIZATION OF TEST AND CONTROL ARTICLES

The test article, WR238605 Succinate, was received by Microbiological Associates, Inc. on August 26, 1994 and was assigned the code number 94BE97. The test article was characterized by the Sponsor as a fine, pale yellow powder that should be stored 2-8°C, in the dark, with no expiration date provided.

Upon receipt, the test article was described as an off-white powder and was stored at 2-8°C, protected from exposure to light. At the time of use, the test article was dissolved in DMSO, obtained from the Fisher Scientific Company.

Mitomycin C (MMC), was obtained from the Sigma Chemical Company, and was dissolved and diluted in sterile distilled water to stock concentrations of 8 and 15 µg/ml. Cyclophosphamide (CP), was obtained from Sigma Chemical Company, and was dissolved and diluted in sterile distilled water to stock concentrations of 1.25 and 2.5 mg/ml.

MATERIALS AND METHODS

Test System

Chinese hamster ovary (CHO-K_1) cells (repository number CCL 61) were obtained from American Type Culture Collection, Rockville, MD. In order to assure the karyotypic stability of the cell line, cells were not used beyond passage 20. The freeze lot of cells was tested using the Hoechst staining procedure and found to be free of mycoplasma contamination.

Activation System

Aroclor 1254-induced rat liver S-9 was used as the metabolic activation system. The S-9 was prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S-9 was batch prepared and stored at ≤-70°C until used. Each bulk preparation of S-9 was assayed for its ability to metabolize 2-aminoanthracene and 7,12-dimethylbenz(α)anthracene to forms mutagenic to *Salmonella typhimurium* TA100.

Immediately prior to use, the S-9 was thawed and mixed with a cofactor pool to contain 2 mM magnesium chloride, 6 mM potassium chloride, 1 mM glucose-6-phosphate, 1 mM nicotinamide adenine dinucleotide phosphate (NADP) and 20 μl S-9 per milliliter medium (McCoy's 5A serum-free medium supplemented with 100 units penicillin and 100 μg streptomycin/ml, and 2 mM L-glutamine).

Preliminary Toxicity Assay

The toxicity test was performed for the purpose of selecting dose levels and harvest times for the chromosome aberration assay and consisted of test article effect on mitotic indices and cell cycle delay. CHO cells were seeded for each treatment condition at approximately 5 x 10^5 cells/25 cm^2 flask and were incubated at 37 ± 1°C in a humidified atmosphere of $5 \pm 1\%$ CO_2 in air for 16-24 hours. Treatment was carried out by refeeding the flasks with 5 ml complete medium (McCoy's 5A medium supplemented with 10% FBS, 100 units penicillin and 100 μg streptomycin/ml, and 2 mM L-glutamine) for the nonactivated study or 5 ml S-9 reaction mixture for the activated study, to which was added 50 μl dosing solution of test article in solvent or solvent alone. The cells were treated for 6 hours in the nonactivated test system and for 4 hours in the S-9 activated system. Two hours after initiation of treatment, a 50 μl aliquot of 1mM 5-bromo-2'-deoxyuridine (BrdU) was added to each flask and incubation continued as required. At completion of the exposure period, the treatment medium was removed, the cells washed with calcium and magnesium-free phosphate buffered saline (CMF-PBS), refed with 5 ml complete medium containing 0.01 mM BrdU and returned to the incubator for a total of 24 hours from the initiation of BrdU treatment. Two hours prior to harvest by trypsinization, Colcemid® was added to each flask at a final concentration of 0.1 μg/ml. Metaphase preparations were prepared and stained for sister chromatid differentiation using a modified fluorescence-plus-Giemsa technique (Perry and Wolff, 1974). Slides were evaluated for the percentage of first, second and third-plus-subsequent-division metaphase cells for estimation of the test article effect on cell cycle kinetics. The average generation time (AGT) was calculated for each treatment condition. The mitotic index was determined for each treatment condition as the percentage of mitotic cells in a population of 500 cells scored.

Definitive Assay

For the chromosome aberration assay, CHO cells were seeded at approximately 5 x 10^5 cells/25 cm^2 flask and were incubated at 37 ± 1°C in a humidified atmosphere of $5 \pm 1\%$ CO_2 in air for 16-24 hours. Treatment was carried out by refeeding duplicate flasks with 5 ml complete medium for the nonactivated study or 5 ml S-9 reaction mixture for the activated study, to which was added 50 μl of dosing solution of test or control article in solvent or solvent alone. An untreated control consisting of cells in complete medium or S-9 reaction mixture was also included.

In the nonactivated study, the cells were exposed continuously for 16 hours at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air. Two hours prior to the scheduled cell harvest, Colcemid® was added to duplicate flasks for each treatment condition at a final concentration of 0.1 μg/ml and the flasks returned to the incubator until cell collection.

In the S-9 activated study, the cells were exposed for 4 hours at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air. After the exposure period, the treatment medium was removed, the cells washed with CMF-PBS, refed with complete medium and returned to the incubator for an additional 14 hours. At this time, Colcemid® was added to duplicate flasks for each treatment condition at a final concentration of 0.1 μg/ml and the flasks incubated for an additional two hours.

Collection of Metaphase Cells

Two hours after the addition of Colcemid®, metaphase cells were harvested for both the nonactivated and S-9 activated studies by trypsinization. Cells were collected approximately 18 and 20 hours after initiation of treatment in the nonactivated and the S-9 activated assay systems, respectively. The cells were collected by centrifugation at approximately 800 rpm for 5 minutes. The cell pellet was resuspended in 2-4 ml 0.075 M potassium chloride (KCl) and allowed to stand at room temperature for 4-8 minutes. The cells were collected by centrifugation, the supernatant aspirated and the cells fixed with two washes of approximately 2 ml Carnoy's fixative (methanol:glacial acetic acid, 3:1, v/v). The cells were stored overnight or longer in fixative at approximately 2-6°C.

Slide Preparation

To prepare slides, the fixed cells were centrifuged at approximately 800 rpm for 5 minutes, the supernatant fluid decanted and the cells resuspended to opalescence in fresh fixative. A sufficient amount of cell suspension was dropped onto the center of a moist glass slide and allowed to air dry overnight. Slides were identified by the study number, date prepared and the treatment condition. The dried slides were stained with 5% Giemsa, air dried and permanently mounted.

Evaluation of Metaphase Cells

Slides were coded using random numbers by an individual not involved with the scoring process. Metaphase cells with 20±2 centromeres were examined under oil immersion without prior knowledge of treatment groups. Whenever possible, a minimum of 100 metaphase spreads (50 per duplicate flask) were examined and scored for chromatid-type and chromosome-type aberrations. Chromatid-type aberrations include chromatid and isochromatid breaks and exchange figures such as quadriradials (symmetrical and asymmetrical interchanges), triradials, and complex

rearrangements. Chromosome-type aberrations include chromosome breaks and exchange figures such as dicentrics and rings. Fragments (chromatid or acentric) observed in the absence of any exchange figure were scored as a break (chromatid or chromosome). Fragments observed with an exchange figure were not scored as an aberration but instead were considered part of the incomplete exchange. Pulverized chromosome(s), pulverized cells and severely damaged cells (≥ 10 aberrations) were also recorded. Chromatid and isochromatid gaps were recorded but not included in the analysis. The XY coordinates for each cell with chromosomal aberrations were recorded using a calibrated microscope stage. The mitotic index was recorded as the percentage of cells in mitosis per 500 cells counted.

Controls

MMC was used as the positive control in the nonactivated study at final concentrations of 0.08 and 0.15 μg/ml. CP was used as the positive control in the S-9 activated study at final concentrations of 12.5 and 25 μg/ml. For both positive controls the dose with sufficient scorable metaphase cells was selected for analysis. The solvent vehicle for the test article was used as the solvent control at the same concentration as that found in the test article-treated groups. Complete medium or S-9 reaction mixture was used in the untreated control.

Evaluation of Test Results

The toxic effects of treatment are based upon mitotic inhibition relative to the solvent-treated control and are presented for the toxicity and aberration studies. The number and types of aberrations found, the percentage of structurally damaged cells (percent aberrant cells) in the total population of cells examined, and the frequency of structural aberrations per cell (mean aberrations per cell) was calculated and reported for each group. Chromatid and isochromatid gaps are presented in the data but are not included in the total percentage of cells with one or more aberrations or in the frequency of structural aberrations per cell.

Statistical analysis of the percent aberrant cells was performed using the Fisher's exact test. Fisher's test was used to compare pairwise the percent aberrant cells of each treatment group with that of the solvent control. In the event of a positive Fisher's test at any test article dose level, the Cochran-Armitage test was used to measure dose-responsiveness.

All conclusions were based on a sound scientific basis; however, as a guide to interpretation of the data, the test article was considered to induce a positive response when the percentages of cells with aberrations are increased in a dose-responsive manner with one or more concentrations being statistically elevated relative to the solvent control group ($p \leq 0.05$). A significant increase at the high dose only with no dose response was considered suspect. A significant increase at one dose level other than the high dose with no dose response was considered

equivocal. The absence of a statistically significant increase in chromosome aberrations in the test article treated groups, relative to the solvent control group, was concluded to be negative.

Criteria for Determination of a Valid Test

The frequency of cells with structural chromosome aberrations in either the untreated or solvent control must be no greater than 6%. The percentage of cells with chromosome aberrations in the positive control must be statistically increased ($p \leq 0.05$, Fisher's exact test) relative to the solvent control or to the untreated control if a solvent other than water was used.

Record and Specimen Archives

All raw data, draft and final reports, and stained and coded slides are maintained in the archives of Microbiological Associates, Inc. located at 9900 Blackwell Road, Rockville, Maryland 20850.

RESULTS AND DISCUSSION

Dose levels for the chromosome aberration assay were selected following a preliminary toxicity test based upon reduction in mitotic index after treatment relative to the solvent control (Tables 1 and 2). CHO cells were exposed to solvent alone and to nine concentrations of test article ranging from 0.022 μg base/ml to 224 μg base/ml in the absence and presence of an S-9 reaction mixture. The test article was soluble in solvent (DMSO) at a stock concentration of 22.4 mg base/ml. In the nonactivated system the test article was partially soluble in treatment medium at the 224 μg base/ml concentration (but was re-classified as soluble after a 2 hour incubation at 37°C), and soluble in treatment medium at all other concentrations tested. In the S-9 activated system the test article was partially soluble at the 22.4, 67.2, and 224 μg/ml concentrations, and soluble at all other concentrations tested. The osmolality of the highest concentration tested, 224 μg base/ml, was 445 mOsm/kg. The osmolality of the solvent was 459 mOsm/kg. The pH of the test article was approximately 7.5 in treatment medium. In the preliminary cytotoxicity test, toxicity (measured by mitotic inhibition relative to the solvent control) was 26% in the nonactivated test system at the 2.24 μg base/ml dose level. There was also an increase in the average generation time (AGT) from approximately 12 hours to approximately 13 hours. In the nonactivated test system, complete mitotic inhibition was seen at dose levels of 6.72 μg base/ml and greater. In the S-9 activated test system toxicity (measured by mitotic inhibition relative to the solvent control) was 47% at the 6.72 μg base/ml dose level. The AGT was increased from approximately 12 hours to approximately 21 hours. Complete mitotic inhibition was seen at dose levels of 22.4 μg base/ml and greater in the S-9 activated system. Based upon the

findings of the toxicity study, dose levels of 1.8, 2.2, 2.8, 3.5, 4.3, and 5.4 μg base/ml were selected for further study in the nonactivated chomosome aberration assay; and 4.5, 5.6, 7, 8.8, 11, 14, and 18 μg base/ml were selected for further study in the S-9 activated chromosome aberration assay. Due to the cell cycle delay (increased AGT) observed in the preliminary toxicity test, the cell harvest times were set at 18 and 20 hours after treatment initiation for the nonactivated and S-9 activated test systems, respectively, to assure microscopic evaluation of first-division metaphase cells.

The activity of WR238605 Succinate in the induction of chromosome aberrations in CHO cells when treated in the absence of an exogenous source of metabolic activation is presented by treatment flask in Table 3 and summarized by group in Table 5. The test article was soluble in solvent at a stock concentration of 0.54 mg base/ml and was soluble in treatment medium at all dose levels tested. Toxicity (mitotic inhibition) was approximately 69% at 3.5 μg/ml, the highest test concentration evaluated for structural chromosome aberrations. Excessive toxicity precluded the analysis of the 4.3 and 5.4 μg base/ml dose levels. The percentage of cells with structural aberrations in the test article-treated groups was not significantly increased above that of the solvent control ($p>0.05$, Fisher's exact test). The percentage of damaged cells in the MMC group was 15% ($p\leq 0.01$, Fisher's exact test).

The activity of WR238605 Succinate in the induction of chromosome aberrations in CHO cells when treated in the presence of an S-9 reaction mixture is presented by treatment flask in Table 4 and summarized by group in Table 5. The test article was soluble in solvent at a stock concentration of 1.8 mg base/ml and was soluble in treatment medium at all dose levels tested. Toxicity (mitotic inhibition) was approximately 72% at 11 μg base/ml, the highest test concentration evaluated for structural chromosome aberrations. Excessive toxicity precluded the analysis of the 14 and 18 μg base/ml dose levels. The 4.5 μg base/ml dose level was tested to ensure four scorable dose levels, but was not needed for microscopic analysis. The percentage of cells with structural aberrations in the test article-treated groups was not statistically increased above that of the solvent control ($p>0.05$, Fisher's exact test). The percentage of damaged cells in the CP group was 37% ($p\leq 0.01$, Fisher's exact test).

CONCLUSION

The positive and negative controls fulfilled the requirements for a valid test.

Under the conditions of the assay described in this report, WR238605 Succinate was concluded to be negative in the chromosome aberration assay using Chinese hamster ovary (CHO) cells .

TABLE 1
PRELIMINARY TOXICITY TEST USING WR238605 Succinate IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

Treatment[1]	Mitotic Index[2] (%)	Percent Change[3]	Cell Cycle Kinetics Percentage of cells in M_1	M_2	M_3	Average Generation Time[4] (AGT)
DMSO	6.8		1	99	0	12.1
WR238605 Succinate						
0.022 µg base/ml	5.4	-21	3	97	0	12.2
0.067 µg base/ml	6.2	-9	2	98	0	12.1
0.224 µg base/ml	6.6	-3	2	98	0	12.1
0.672 µg base/ml	7.0	3	5	95	0	12.3
2.24 µg base/ml	5.0	-26	15	85	0	13.0
6.72 µg base/ml	0.0[5]	-100	--[6]	--	--	--
22.4 µg base/ml	0.0[5]	-100	--[6]	--	--	--
67.2 µg base/ml	0.0[5]	-100	--[6]	--	--	--
224 µg base/ml	0.0[5]	-100	--[6]	--	--	--

[1]CHO cells were treated in the absence of an exogenous source of metabolic activation for 6 hours at 37±1°C. Metaphase cells were collected following a 24 hour growth period in BrdU.
[2]Mitotic Index = (Cells in mitosis/500 cells scored) x 100.
[3]Percent change = (Treatment mitotic index - control mitotic index)/control mitotic index, expressed as a percentage.
[4]Average Generation Time:

$$\frac{\text{24 hours of BrdU exposure}}{[(1 \times \text{frequency M1 cells}) + (2 \times \text{frequency M2 cells}) + (3 \times \text{frequency M3 cells})]}$$

[5]Complete mitotic inhibition.
[6]Unable to score due to excessive toxicity.

TABLE 2
PRELIMINARY TOXICITY TEST USING WR238605 Succinate IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

Treatment[1]	Mitotic Index[2] (%)	Percent Change[3]	Cell Cycle Kinetics Percentage of cells in M_1	M_2	M_3	Average Generation Time[4] (AGT)
DMSO	6.4		4	96	0	12.2
WR238605 Succinate						
0.022 µg base/ml	6.2	-3	4	96	0	12.2
0.067 µg base/ml	7.0	9	2	98	0	12.1
0.224 µg base/ml	6.4	0	9	91	0	12.6
0.672 µg base/ml	7.2	12	7	93	0	12.4
2.24 µg base/ml	5.2	-19	84	16	0	20.7
6.72 µg base/ml	3.4	-47	85	15	0	20.9
22.4 µg base/ml	0.0[5]	-100	--[6]	--	--	--
67.2 µg base/ml	0.0[5]	-100	--[6]	--	--	--
224 µg base/ml	0.0[5]	-100	--[6]	--	--	--

[1]CHO cells were treated in the presence of an exogenous source of metabolic activation for 4 hours at 37±1°C. Metaphase cells were collected following a 24 hour growth period in BrdU.
[2]Mitotic Index = (Cells in mitosis/500 cells scored) x 100.
[3]Percent change = (Treatment mitotic index - control mitotic index)/control mitotic index, expressed as a percentage.
[4]Average Generation Time:

$$\frac{\text{24 hours of BrdU exposure}}{[(1 \times \text{frequency M1 cells}) + (2 \times \text{frequency M2 cells}) + (3 \times \text{frequency M3 cells})]}$$

[5]Complete mitotic inhibition.
[6]Unable to score due to excessive toxicity.

TABLE 3
CYTOGENETIC ANALYSIS OF CHO CELLS TREATED WITH WR238605 Succinate IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

Treatment[1]	Flask	Mitotic Index[2] (%)	Cells Scored	Aberrant Cells[3] (%)	Total Number of Structural Aberrations: Chromatid-type[4] Gaps	Chromatid-type[4] Breaks	Chromatid-type[4] Exch	Chromosome-type[5] Breaks	Chromosome-type[5] Dic	Chromosome-type[5] Ring	Severely Damaged Cells[6]	Average Aberrations Per Cell[3,7]
18 Hour Harvest												
Untreated cells	A	4.8	50	0	0	0	0	0	0	0	0	0.000
	B	5.6	50	0	0	0	0	0	0	0	0	0.000
DMSO	A	4.2	50	0	0	0	0	0	0	0	0	0.000
	B	6.8	50	0	1	0	0	0	0	0	0	0.000
WR238605 Succinate 1.8 µg base/ml	A	4.0	50	0	0	0	0	0	0	0	0	0.000
	B	5.4	50	2	0	1	0	0	0	0	0	0.020
2.2 µg base/ml	A	5.2	50	0	0	0	0	0	0	0	0	0.000
	B	4.0	50	0	0	0	0	0	0	0	0	0.000
2.8 µg base/ml	A	2.8	50	0	0	0	0	0	0	0	0	0.000
	B	3.8	50	0	1	0	0	0	0	0	0	0.000
3.5 µg base/ml	A	1.6	50	0	0	0	0	0	0	0	0	0.000
	B	1.8	50	0	0	0	0	0	0	0	0	0.000
MMC 0.08 µg/ml	A	5.0	50	12	0	7	0	0	0	0	0	0.140
	B	4.2	50	18	1	12	0	0	0	0	0	0.240

[1]CHO cells were treated for 18 hours at 37±1°C in the absence of an exogenous source of metabolic activation.
[2]Mitotic index = number mitotic figures x 100/500 cells counted.
[3]Excluding cells with only gaps.
[4]Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures include quadriradials, triradials and complex rearrangements.
[5]Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.
[6]Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.
[7]Severely damaged cells and pulverizations were counted as 10 aberrations.

TABLE 4

CYTOGENETIC ANALYSIS OF CHO CELLS TREATED WITH WR238605 Succinate IN THE PRESENCE OF EXOGENOUS METABOLIC ACTIVATION

Treatment[1]	Flask	Mitotic Index[2] (%)	Cells Scored	Aberrant Cells[3] (%)	Total Number of Structural Aberrations: Chromatid-type[4] Gaps	Chromatid-type[4] Breaks	Chromatid-type[4] Exch	Chromosome-type[5] Breaks	Chromosome-type[5] Dic	Chromosome-type[5] Ring	Severely Damaged Cells[6]	Average Aberrations Per Cell[3,7]
20 Hour Harvest												
Untreated cells	A	9.8	50	0	0	0	0	0	0	0	0	0.000
	B	8.4	50	0	1	0	0	0	0	0	0	0.000
DMSO	A	7.0	50	0	0	0	0	0	0	0	0	0.000
	B	5.8	50	0	1	0	0	0	0	0	0	0.000
WR238605 Succinate 5.6 µg base/ml	A	6.6	50	2	1	1	0	0	0	0	0	0.020
	B	6.8	50	2	5	1	0	0	0	0	0	0.020
7 µg base/ml	A	5.8	50	0	1	0	0	0	0	0	0	0.000
	B	6.0	50	0	0	0	0	0	0	0	0	0.000
8.8 µg base/ml	A	3.0	50	4	3	2	0	0	0	0	0	0.040
	B	4.0	50	2	0	1	0	0	0	0	0	0.020
11 µg base/ml	A	1.6	50	8	3	5	0	0	0	0	0	0.100
	B	2.0	50	0	1	0	0	0	0	0	0	0.000
CP, 12.5 µg/ml	A	2.6	50	34	1	28	6	2	0	0	0	0.720
	B	3.2	50	40	2	27	4	2	0	0	0	0.660

[1]CHO cells were treated for 4 hours at 37±1°C in the presence of an exogenous source of metabolic activation.
[2]Mitotic index = number mitotic figures x 100/500 cells counted.
[3]Excluding cells with only gaps.
[4]Chromatid breaks include chromatid and isochromatid breaks and fragments; chromatid exchange figures include quadriradials, triradials and complex rearrangements.
[5]Chromosome breaks include breaks and acentric fragments; dic, dicentric chromosome.
[6]Severely damaged cells includes cells with one or more pulverized chromosomes and cells with 10 or more aberrations.
[7]Severely damaged cells and pulverizations were counted as 10 aberrations.

TABLE 5
SUMMARY

Treatment	S-9 Activation	Harvest Time (hrs)	Mitotic Index	Cells Scored	Aberrations Per Cell[1] (Mean ± SD)	Cells With Aberrations[2] (%)
Untreated	-	18	5.2	100	0.000 ± 0.000	0
DMSO	-	18	5.5	100	0.000 ± 0.000	0
WR238605 Succinate						
1.8 µg base/ml	-	18	4.7	100	0.010 ± 0.100	1
2.2 µg base/ml	-	18	4.6	100	0.000 ± 0.000	0
2.8 µg base/ml	-	18	3.3	100	0.000 ± 0.000	0
3.5 µg base/ml	-	18	1.7	100	0.000 ± 0.000	0
MMC,0.08 µg/ml	-	18	4.6	100	0.190 ± 0.506	15**
Untreated	+	20	9.1	100	0.000 ± 0.000	0
DMSO	+	20	6.4	100	0.000 ± 0.000	0
WR238605 Succinate						
5.6 µg base/ml	+	20	6.7	100	0.020 ± 0.141	2
7 µg base/ml	+	20	5.9	100	0.000 ± 0.000	0
8.8 µg base/ml	+	20	3.5	100	0.030 ± 0.171	3
11 µg base/ml	+	20	1.8	100	0.050 ± 0.261	4
CP, 12.5 µg/ml	+	20	2.9	100	0.690 ± 1.169	37**

[1] Severely damaged cells were counted as 10 aberrations.
[2] **, p≤0.01; Fisher's exact test.

REFERENCES

Evans, H.J. (1976) Cytological methods for detecting chemical mutagens, in: A. Hollaender (Ed.), Chemical Mutagens, Principles and Methods for their Detection, vol 4. Plenum Press, New York.

Perry, P., and S. Wolff (1974) New Giemsa method for differential staining of sister chromatids, Nature, 251:156-158.

Preston, R.J., W. Au, M.A. Bender, J.G. Brewen, A.V. Carrano, J.A. Heddle, A.F. McFee, S. Wolff and J.S. Wassom (1981) Mammalian *in vivo* and *in vitro* cytogenetic assays: a report of the Gene-Tox Program, Mutation Research, 87:143-188.

APPENDIX I

Study Protocol

PROTOCOL AMENDMENT I

SPONSOR:	**Toxicology Research Laboratory**
TEST ARTICLE I.D.:	**WR238605 Succinate**
MA STUDY NO:	**G94BE97.330 (Protocol No.: SPGT330)**
SPONSOR PROJECT NO.:	**UIC-10**
PROTOCOL TITLE:	**Chromosome Aberrations in Chinese Hamster Ovary (CHO) Cells**

1. **LOCATION:** Page 1, § 3.1;

   AMENDMENT: Change storage temperature to 2-6°C.

   REASON FOR THE AMENDMENT: To be consistent with the Standard Operating Procedures of the testing facility.

APPROVALS:

Patrick T. Curry
STUDY DIRECTOR

9-9-94
DATE

SPONSOR REPRESENTATIVE

9/26/94
DATE

MICROBIOLOGICAL ASSOCIATES, INC.

PROTOCOL AMENDMENT II

SPONSOR: **Toxicology Research Laboratory**

TEST ARTICLE I.D.: **WR238605 Succinate**

MA STUDY NO: **G94BE97.330 (Protocol No.: SPGT330)**

SPONSOR PROJECT NO.: **UIC-10**

PROTOCOL TITLE: **Chromosome Aberrations in Chinese Hamster Ovary (CHO) Cells**

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1. **LOCATION:** Page 1, § 3.2; Controls

   AMENDMENT: Amend Positive controls to read "Mitomycin C (MMC)" and "Cyclophosphamide (CP)".

   REASON FOR THE AMENDMENT: The supply of triethylenemelamine (TEM) has run out and is no longer manufactured.

2. **LOCATION:** Page 4 of 8, § 7.5.3; Positive Controls

   AMENDMENT: Amend first sentence to read "Mitomycin C will be used at a concentration within 0.05-0.3 μg/ml as the positive control in the non-activated study.".

   REASON FOR THE AMENDMENT: To specify the range in which MMC is to be used.

APPROVALS:

Patrick T. [illegible] — 10-5-94

STUDY DIRECTOR — DATE

[illegible] — 10/10/94

SPONSOR REPRESENTATIVE — DATE

MICROBIOLOGICAL ASSOCIATES, INC.

APPROVED
CC 9-2-94

Received by RA/QA 9-1-94

MA Study Number: G94BE97.330

CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY (CHO) CELLS

1.0 PURPOSE

The purpose of this study is to evaluate the clastogenic potential of a test article based upon its ability to induce chromosome aberrations in Chinese hamster ovary (CHO) cells.

2.0 SPONSOR

2.1 Name: **Toxicology Research Laboratory**

2.2 Address: University of Illinois at Chicago
Department of Pharmacology
1940 West Taylor Street
Chicago, IL 60612-7353

2.3 Representative: Barry S. Levine, D.Sc., D.A.B.T.

2.4 Sponsor Project #: UIC-10. This study will be conducted by Microbiological Associates, Inc. and is being sponsored by the Toxicology Research Laboratory, UIC under a contract from U.S. Army Medical Research Acquisition Activity (No. DAMD 17-92-C-2001). Dr. George Schieferstein, Contracting Officer's Representative.

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1 Test Article: **WR238605 Succinate**

Storage: 0 - 4°C
Ambient humidity conditions
Protect from light

3.2 Controls: Negative: Untreated Cells
Solvent: Test Article Solvent
Positive: Triethylenemelamine (TEM)
Cyclophosphamide (CP)

3.3 Determination of Strength, Purity, etc.

The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article and the stability and strength of the dosing solutions.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1 Name: Division of Genetic and Cellular Toxicology Microbiological Associates, Inc.

4.2 Address: 9900 Blackwell Road Rockville, MD 20850

4.3 Study Director: Patrick T. Curry, Ph.D.

4.4 Associate Study Director: Donald L. Putman, Ph.D.

5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date: 9-19-94

5.2 Proposed Experimental Completion Date: 11-2-94

5.3 Proposed Report Date: 11-11-94

6.0 TEST SYSTEM

The CHO-K_1 cell line is a proline auxotroph with a modal chromosome number of 20 and a population doubling time of 10-14 hours. CHO-K_1 cells were obtained from the American Type Culture Collection (repository number CCL 61), Rockville, MD. This system has been demonstrated to be sensitive to the clastogenic activity of a variety of chemicals.

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The assay will be performed by exposing CHO cells to a minimum of four concentrations of the test article as well as negative and positive controls in duplicate cultures. In the nonactivated test system, treatment will be continuous up to the time of cell collection; in the S-9 activated test system, exposure will be for 4 hours. The dividing cells will be arrested in metaphase and harvested for microscopic evaluation of chromosome aberrations at approximately 10 hours after the initiation of treatment. In order to ensure evaluation of first-division metaphase cells, the cell collection time may be delayed up to 20 hours in the event of cell cycle delay associated with test article treatment. The clastogenic potential of the test article will be measured by its ability to increase structural chromosome aberrations in a dose-responsive manner when compared to the solvent control group.

7.1 Solubility Determination

Unless the Sponsor has indicated the test article solvent, a solubility determination will be conducted to determine the solvent and the maximum soluble concentration up to a maximum of 500 mg/ml. Vehicles compatible

with this test system, in order of preference, include but are not limited to deionized, distilled water (CAS 7732-18-5), dimethylsulfoxide (CAS 67-68-5), ethanol (CAS 64-17-5), and acetone (CAS 67-64-1). The vehicle will be the solvent, selected in order of preference, that permits preparation of the highest stock concentration, up to 500 mg/ml.

7.2 Dose Levels

Selection of the dose levels for the cytogenetics assay will be based upon mitotic inhibition after treatment. CHO cells will be exposed to solvent alone and to at least nine concentrations of test article, the highest concentration being the limit of solubility but not to exceed 5 mg/ml. The pH will be measured at the highest test article treatment condition and will be adjusted, if necessary, in order to maintain a neutral pH in the treatment medium. The osmolality of the highest treatment condition in treatment medium will also be measured. Cells seeded 16-24 hours earlier will be exposed for 6-8 hours in the absence of S-9 and for 4 hours in the presence of S-9. Two hours after initiation of exposure, 5-bromo-2'-deoxyuridine (BrdU), will be added to the culture medium at a final concentration of 0.01 mM. Following a 24 hour growth period in medium containing BrdU, with Colcemid® (0.1 μg/ml) present for the last 2 hours, the cells will be harvested by trypsinization. Metaphase preparations will be made and stained for sister chromatid differentiation using a modified fluorescence-plus-Giemsa technique (Perry and Wolff, 1974). Slides will be evaluated for the percentage of first-, second- and third-plus-subsequent-division metaphase cells per 100 cells scored for determination of the test article effect on cell cycle kinetics. The average generation time (AGT) of each treatment condition will be calculated. The percentage of cells in mitosis per 500 cells scored (mitotic index) will be determined for each treatment group.

Whenever possible, the high dose will be selected to give at least 50% toxicity (mitotic inhibition). At least three additional dose levels will be included in the assay. In the event the test article cannot be dissolved at a high enough concentration in an appropriate solvent to be toxic, if excessive precipitation of the test article-solvent solution occurs upon addition to treatment medium, or if osmolality of the treatment medium is excessive, the Sponsor will be consulted prior to dose selection. The AGT will be used to determine the optimum harvest time for the cytogenetic study.

7.3 Frequency and Route of Administration

Target cells will be treated for 10 hours in the absence of S-9 (or up to 20 hours depending upon cell cycle delay) and for 4 hours in the presence of S-9 by incorporation of the test article-solvent mixture into the treatment medium. This technique has demonstrated to be an effective method of detection of chemical clastogens in this test system.

7.4 Activation System

Aroclor 1254-induced rat liver S-9 will be used as the metabolic activation system. The S-9 will be prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, five days prior to sacrifice. The S-9 will be batch prepared and stored frozen at approximately -70°C until used.

Immediately prior to use, the S-9 will be thawed and mixed with cofactors to contain 2 mM magnesium chloride ($MgCl_2$,) 6 mM potassium chloride (KCl), 1mM glucose-6-phosphate, 1 mM nicotinamide adenine dinucleotide phosphate (NADP) and 20 µl S-9 per ml serum free medium.

7.5 Controls

7.5.1 Untreated Control

Untreated cells will be used as the untreated control.

7.5.2 Solvent Control

The solvent vehicle for the test article will be used as the solvent control. For solvents other than water or medium, the final concentration in treatment medium will not exceed 1%.

7.5.3 Positive Controls

Triethylenemelamine will be used at a concentration within 0.25-1 µg/ml as the positive control in the non-activated study. Cyclophosphamide will be used at a concentration within 10-50 µg/ml as the positive control in the S-9 activated study.

7.6 Preparation of Target Cells

Exponentially growing CHO-K_1 cells will be seeded in complete medium (McCoy's 5A medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 units penicillin/ml and 100 µg streptomycin/ml) for each treatment condition at approximately 5×10^5 cells/25 cm^2 flask. The flasks will be incubated at 37 $\pm$ 1°C in a humidified atmosphere of 5 $\pm$ 1% CO_2 in air for 16-24 hours.

7.7 Identification of Test System

Using a permanent marking pen, the treatment flasks will be identified by the MA study number and a code system to designate the treatment condition and test phase.

7.8 Treatment of Target Cells

Treatment will be carried out in duplicate by refeeding the flasks with 5 ml complete medium for the nonactivated exposure or 5 ml S-9 reaction mixture for the S-9 activated exposure, to which will be added 50 μl of dosing solution of test or control article in solvent or solvent alone. Larger volumes of dosing solution may be used if water or medium is used as the solvent. An untreated control consisting of cells in complete medium or S-9 reaction mixture will also be included.

In the nonactivated study, the cells will be treated for 10 hours at 37 $\pm$ 1°C in a humidified atmosphere of 5 $\pm$ 1% CO_2 in air. Treatment will be extended to up to 20 hours in those dose levels with considerable cell cycle delay. Treatment will continue until collection of metaphase cells.

In the S-9 activated study, the cells will be treated for 4 hours at 37 $\pm$ 1°C in a humidified atmosphere of 5 $\pm$ 1% CO_2 in air. After the treatment period, the treatment medium will be aspirated, the cells washed with phosphate buffered saline, refed with complete medium and returned to the incubator.

7.9 Collection of Metaphase Cells

Cells will be collected approximately 10 hours after initiation of treatment or at a later time selected to represent the first division metaphase after initiation of treatment if the test article causes considerable cell cycle delay. Cell fixation will not extend beyond 20 hours after initiation of treatment. Two hours prior to harvest, Colcemid® will be added to the cultures at a final concentration of 0.1 μg/ml.

Cells will be harvested by trypsinization, collected by centrifugation, swollen with 0.075M KCl, washed with two consecutive changes of fixative (methanol:glacial acetic acid, 3:1 v/v), capped and stored overnight or longer at approximately 2-6°C. To prepare slides, the cells will be collected by centrifugation and resuspended in fresh fixative. One to two drops of fixed cells will be dropped onto a glass microscope slide and air-dried. The slide will be identified by the experiment number, treatment condition and date. One to two slides will be prepared from each treatment flask. The slides will be stained with Giemsa and permanently mounted.

7.10 Scoring for Metaphase Aberrations

Slides will be coded using random numbers by an individual not involved with the scoring process. Metaphase cells with 20 $\pm$ 2 centromeres will be examined under oil immersion without prior knowledge of treatment groups. Whenever possible, a minimum of 100 metaphase spreads from each dose level (50 per duplicate flask) will be examined and scored for chromatid-type

and chromosome-type aberrations. Chromatid-type aberrations include chromatid and isochromatid breaks and exchange figures such as quadriradials (symmetrical and asymmetrical interchanges), triradials, and complex rearrangements. Chromosome-type aberrations include chromosome breaks and exchange figures such as dicentrics and rings. Fragments (chromatid or acentric) observed in the absence of any exchange figure will be scored as a break (chromatid or chromosome). Fragments observed with an exchange figure will not be scored as an aberration but will be considered part of the incomplete exchange. Pulverized chromosome(s), pulverized cells and severely damaged cells ($\geq$ 10 aberrations) will also be recorded. Chromatid and isochromatid gaps will be recorded but not included in the analysis. The XY coordinates for each cell with a structural aberration will be recorded using a calibrated microscope stage. The mitotic index will be recorded as the percentage of cells in mitosis per 500 cells counted.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

8.1 Negative Controls

The frequency of cells with structural chromosome aberrations in either the untreated or solvent control must be no greater than 6%.

8.2 Positive Control

The percentage of cells with aberrations must be statistically increased ($p \leq 0.05$, Fisher's exact test) relative to the solvent control or to the untreated control if a solvent other than water was used.

9.0 EVALUATION OF TEST RESULTS

The toxic effects of treatment are based upon mitotic inhibition relative to the solvent-treated control and will be presented for the toxicity and aberration study. The AGT will be calculated for each treatment condition in the toxicity study as: AGT = (24 hours x 100)/[(number M_1 cells x 1)+(number M_2 cells x 2)+(number M_3 cells x 3)]. The number and types of aberrations found, the percentage of structurally damaged cells in the total population of cells examined, and the mean aberrations per cell will be calculated and reported for each treatment group. Chromatid and isochromatid gaps are presented in the data but are not included in the total percentage of cells with one or more aberrations or in the frequency of structural aberrations per cell. Statistical analysis of the percentage of aberrant cells will be performed using the Fisher's exact test. The Fisher's test will be used to compare pairwise the percent aberrant cells of each treatment group with that of the solvent control. In the event of a positive Fisher's exact test at any test article dose level, the Cochran-Armitage test will be used to measure dose-responsiveness. All conclusions will be based on sound scientific basis; however, as a guide to interpretation of the data, the test article will be considered to induce a positive

response when the percentage of cells with aberrations is increased in a dose-responsive manner with one or more concentrations being statistically significant ($p \leq 0.05$). A significant increase at the high dose only with no dose response will be considered suspect. A significant increase at one dose level other than the high dose with no dose response will be considered equivocal. Test articles not demonstrating a statistically significant increase in aberrations will be concluded to be negative.

10.0 REPORT

A report of the results of this study will be prepared by Microbiological Associates, Inc. and will accurately describe all methods used for generation and analysis of the data.

Results presented will include , but not be limited to:

- cells used
- test conditions: composition of medium, CO_2 concentration, incubation time, concentration of test article and rationale for selection of concentration, duration of treatment, duration of treatment with and concentration of Colcemid®, type of metabolic activation system used, positive and negative controls
- number of cell cultures
- number of metaphases analyzed (method for determination; data given separately for each culture)
- mitotic index
- criteria for scoring aberrations
- type and number of aberrations, given separately for each treated and control culture
- historical control data

11.0 RECORDS AND ARCHIVES

11.1 Records

Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates, Inc., 9900 Blackwell Road, Rockville, MD, 20850, in accordance with the relevant Good Laboratory Practice Regulations.

11.2 Specimens

All specimens, such as microscope slides, will be held in storage as long as the quality of the preparation affords evaluation or in accordance with the relevant Good Laboratory Practice Regulations.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol was designed to fulfill EPA and OECD test guidelines.

This study will be performed in compliance with the provisions of the Good Laboratory Practice Regulations For Nonclinical Laboratory Studies.

Will this study be submitted to a regulatory agency? Yes

If so, to which agency or agencies? US FDA

Unless arrangements are made to the contrary, the Test Article will be returned to the Sponsor at the conclusion of the study. In addition, does the Sponsor request that samples of the Test Article dosing solutions be returned? Yes

13.0 REFERENCES

Evans, H.J. (1976) Cytological methods for detecting chemical mutagens, in: A. Hollaender (Ed.), Chemical Mutagens, Principles and Methods for their Detection, vol. 4. Plenum Press, New York, NY.

Perry, P. and S. Wolff (1974) New Giemsa method for differential staining of sister chromatids, Nature, 251: 156-158.

Preston, R.J., W. Au, M.A. Bender, J.G. Brewen, A.V. Carrano, J.A. Heddle, A.F. McFee, S. Wolff and J.S. Wassom (1981) Mammalian in vivo and in vitro cytogenetic assays: a report of the Gene-Tox Program, Mutation Research, 87:143-188.

14.0 APPROVAL

Barry S. Levine — 6/29/94

Barry S. Levine, D.Sc., D.A.B.T. (Sponsor Representative) — Date

G Schieferstein — 7/5/9X.

Dr. George Schieferstein (US Army Contracting Officer's Representative) — Date

Patrick T. Curry — 8-31-94

MA Study Director — Date

APPENDIX II

Historical Control Data

IN VITRO MAMMALIAN CYTOGENETIC TEST USING CHINESE HAMSTER OVARY (CHO) CELLS

HISTORICAL CONTROL VALUES
1991-1993

NONACTIVATED TEST SYSTEM

Historical Values	Aberrant Cells	
	Untreated Control	Solvent Control[1]
Mean	1.3%	1.5%
Standard Deviation	1.1%	1.2%
Range	0.0% to 4.5%	0.0% to 6.0%

S-9 ACTIVATED TEST SYSTEM

Historical Values	Aberrant Cells	
	Untreated Control	Solvent Control[1]
Mean	1.5%	1.6%
Standard Deviation	1.2%	1.1%
Range	0.0% to 5.5%	0.0% to 4.5%

[1]Solvents include water, saline, dimethylsulfoxide, ethanol, acetone, toluene, CMC, PBS, 2% DMSO in saline, CSEP Buffer

APPENDIX 3

CHO/HGPRT MUTATION ASSAY REPORT

FINAL REPORT

Study Title

CHO/HGPRT MUTATION ASSAY

Test Article

WR238605 Succinate

Authors

Richard H. C. San, Ph.D.
Jane J. Clarke, B.A.

Study Completion Date

January 4, 1996

Performing Laboratory

Microbiological Associates, Inc.
9900 Blackwell Road
Rockville, MD 20850

Laboratory Study Number

G94BE97.782

Sponsor Project Number

UIC-10

Sponsor

Toxicology Research Laboratory
University of Illinois at Chicago
Department of Pharmacology
1940 West Taylor Street
Chicago, IL 60612-7353

MICROBIOLOGICAL ASSOCIATES, INC.

STATEMENT OF COMPLIANCE

Microbiological Associates, Inc.'s Study No. G94BE97.782, was conducted in compliance with the U.S. FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the U.S. EPA GLP Standards 40 CFR 160 and 40 CFR 792, the UK GLP Compliance Programme, the Japanese GLP Standard and the OECD Principles of Good Laboratory Practice in all material aspects with the following exceptions:

> The identity, strength, purity and composition or other characteristics to define the test or control article were not determined by the testing facility.
>
> The stability of the test or control article under the test conditions was not determined by the testing facility.
>
> Analyses to determine the uniformity, concentration, or stability of the test or control mixtures were not performed by the testing facility.

Richard

Richard H. C. San, Ph.D.
Study Director

1/4/96

Date

QUALITY ASSURANCE STATEMENT

Study Title: CHO/HGPRT MUTATION ASSAY

Study Number: G94BE97.782

Study Director: Richard H. C. San, Ph.D.

This study has been divided into a series of in-process phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment records, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), the UK GLP Compliance Programme, the Japanese GLP Standard, and the OECD Principles of Good Laboratory Practice and to assure that the study is conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 09 FEB 95, TO STUDY DIR 09 FEB 95, TO MGMT 09 FEB 95
PHASE: Protocol Review

INSPECT ON 01 MAR 95, TO STUDY DIR 01 MAR 95, TO MGMT 03 MAR 95
PHASE: Preparation of cell cultures

INSPECT ON 05 APR 95-07 APR 95, TO STUDY DIR 07 APR 95, TO MGMT 11 APR 95
PHASE: Draft Report

INSPECT ON 28 APR 95, TO STUDY DIR 28 APR 95, TO MGMT 28 APR 95
PHASE: Draft to Revised Draft Report

INSPECT ON 05 JAN 96, TO STUDY DIR 05 JAN 96, TO MGMT 05 JAN 96
PHASE: Revised Draft to Final Report

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Diane B Madsen — 1-5-96

Diane B. Madsen
QUALITY ASSURANCE — DATE

CHO/HGPRT MUTATION ASSAY

FINAL REPORT

Sponsor: **Toxicology Research Laboratory**
University of Illinois at Chicago
Department of Pharmacology
1940 West Taylor Street
Chicago, IL 60612-7353

Authorized Representative: **Barry S. Levine, D.Sc., D.A.B.T.**

Performing Laboratory: **Microbiological Associates, Inc. (MA)**
9900 Blackwell Road
Rockville, Maryland 20850

Test Article I.D.: **WR238605 Succinate**

Test Article Bottle No.: **BM12562**

Test Article Purity: **>99.9% (Provided by Sponsor)**

Test Article Base Mole Fraction: **0.8**

Sponsor Project No.: **UIC-10**

MA Study No.: **G94BE97.782**

Test Article Description: **off-white powder**

Storage Conditions: **2-8°C; protected from exposure to light and moisture**

Test Article Receipt: **August 26, 1994**

Study Initiation: **February 9, 1995**

Laboratory Manager: Jane J. Clarke, B.A.

Study Director: Richard H. C. San, Ph.D. 1/4/96
Date

TABLE OF CONTENTS

SUMMARY

The test article, WR238605 Succinate, was tested in the CHO/HGPRT Mutation Assay in the absence and presence of Aroclor-induced rat liver S9. The assay was performed in two phases. The first phase, the preliminary toxicity assay, was used to establish the dose range for the mutagenesis assay. The second phase, the mutagenesis assay, was used to evaluate the mutagenic potential of the test article. The dosing solutions were adjusted to compensate for the base mole fraction of the test article.

Dimethylsulfoxide (DMSO) was determined to be the solvent of choice based on solubility of the test article and compatibility with the target cells. The test article was soluble in DMSO at a maximum concentration of approximately 250 mg base/ml.

In the preliminary toxicity assay, the maximum concentration of WR238605 Succinate tested was 5000 μg base/ml; this dose was delivered as a suspension from a stock of 500 mg base/ml DMSO into treatment medium. All other doses were delivered as solutions in DMSO into treatment medium. Visible precipitate was observed in treatment medium at concentrations of 1500 and 5000 μg base/ml. Treatment medium was cloudy but with no visible precipitate at concentrations of 50, 150 and 500 μg base/ml. Concentrations of $\leq$15 μg base/ml were soluble in treatment medium. Selection of dose levels for the mutagenesis assay was based on the cloning efficiency relative to the solvent control. Substantial toxicity, i.e., cloning efficiency $\leq$50% of the solvent control, was observed at dose levels $\geq$5.0 μg base/ml without activation and $\geq$15 μg base/ml with S9 activation. Based on these findings, the doses chosen for the mutagenesis assay ranged from 1.0 to 4.0 μg base/ml for the non-activated cultures and 2.5 to 10 μg base/ml for the S9-activated cultures.

In the first trial of the mutagenesis assay, after plating for concurrent cytotoxicity, all the cultures exhibited complete loss of cells during the expression period and were not cloned for mutant selection. The mutagenesis assay was repeated. Based on the concurrent cytotoxicity data from the first trial, the lower dose levels were essentially non-cytotoxic; therefore, a narrower dose range with focus on cytotoxic dose levels was selected for the second trial. The doses chosen for the second trial ranged from 2.0 to 4.0 μg base/ml for the non-activated cultures and from 6.0 to 10 μg base/ml for the S9-activated cultures. All concentrations of test article were delivered as solutions in DMSO into treatment medium and all concentrations remained soluble in treatment medium. In the mutagenesis assay, no positive responses, i.e., treated cultures with mutant frequencies >40 mutants per 10^6 clonable cells, were observed. Toxicity, i.e., cloning efficiency $\leq$50% of the solvent control, was observed only at 9.0 μg base/ml with S9 activation.

Under the conditions of this study, test article WR238605 Succinate was concluded to be negative in the CHO/HGPRT Mutation Assay.

PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test article based on quantitation of forward mutations at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus of Chinese hamster ovary (CHO) cells.

CHARACTERIZATION OF TEST AND CONTROL ARTICLES

The test article, WR238605 Succinate, was received by Microbiological Associates, Inc. on August 26, 1994 and was assigned the code number 94BE97. The test article was characterized by the Sponsor as a fine, pale yellow powder, which should be stored refrigerated in the dark. A purity of >99.9% and base mole fraction of 0.8 were provided by the Sponsor. Upon receipt, the test article was described as an off-white powder and was stored at 2-8°C, protected from exposure to light and moisture.

The vehicle used to deliver WR238605 Succinate was DMSO (CAS 67-68-5) obtained from Fisher Scientific Company. The dosing solutions were adjusted to compensate for the base mole fraction of the test article. Aliquots of the dosing solution preparations from each trial of the mutagenesis assay will be returned to the Sponsor for chemical analysis at the conclusion of the study.

Ethyl methanesulfonate (EMS), CAS 62-50-0, was obtained from Eastman Kodak Chemical Company and was diluted to a stock concentration of 20 μl/ml for use as the positive control for the non-activated test system. Benzo(a)pyrene (B(a)P) was obtained from Sigma Chemical Company and was diluted to stock concentrations of 400 and 500 μg/ml for use as the positive control for the S9-activated test system.

MATERIALS AND METHODS

Test System

CHO-K_1-BH_4 cells were obtained from Dr. Abraham Hsie, Oak Ridge National Laboratories, Oak Ridge, TN. CHO cells were cleansed in medium supplemented with hypoxanthine, aminopterin and thymidine (HAT) then frozen. The freeze lot of cells was tested and found to be free of mycoplasma contamination. Cells used in the mutation assay were within four subpassages from frozen stock in order to assure karyotypic stability.

Metabolic Activation System

Aroclor 1254-induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor-1254, 500 mg/kg, five days prior to sacrifice. The S9 was batch prepared and stored at $\leq$ -70°C until used. Each bulk preparation of S9 was assayed

for sterility and its ability to metabolize 2-aminoanthracene and 7,12-dimethylbenz(a)anthracene to forms mutagenic to *Salmonella typhimurium* TA100.

Immediately prior to use, the S9 reaction mixture was prepared by mixing S9 and 10 mM calcium chloride ($CaCl_2$) with a filter-sterilized cofactor pool to contain 100 μl S9/ml cofactor pool, 4 mM nicotinamide adenine dinucleotide phosphate (NADP), 5 mM glucose-6-phosphate, 30 mM potassium chloride (KCl), 10 mM magnesium chloride ($MgCl_2$), and 50 mM sodium phosphate buffer, pH 8.0. The S9 reaction mixture was stored on ice until used.

Solubility Test

A solubility test was conducted to select the vehicle. The test was conducted using one or more of the following solvents in the order of preference as listed: distilled water, dimethylsulfoxide, ethanol and acetone. The test article was tested to determine the vehicle, selected in order of preference, that permitted preparation of the highest soluble or workable stock concentration, up to 500 mg/ml.

Preliminary Toxicity Assay

The preliminary toxicity assay was used to establish the optimal dose levels for the mutagenesis assay and consisted of evaluation of test article effect on colony-forming efficiency. CHO cells were exposed for 5 hours at 37±1°C to the vehicle alone and nine concentrations of test article ranging from 0.5 to 5000 μg base/ml in both the absence and presence of S9-activation.

Mutagenesis Assay

The mutagenesis assay was used to evaluate the mutagenic potential of the test article. CHO cells were exposed for 5 hours at 37±1°C to the vehicle alone, appropriate positive controls and at least five concentrations of test article in duplicate in both the absence and presence of S9.

Treatment of the Target Cells

The mutagenesis assay was performed according to a protocol developed from published methodologies (Hsie *et al.*, 1981; and O'Neill *et al.*, 1977). Exponentially growing CHO-K_1-BH_4 cells were seeded in F12FBS5-Hx at a density of $5x10^5$ cells/25 cm^2 flask and were incubated at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air for 18-24 hours. F12FBS5-Hx is Ham's F12 medium without hypoxanthine supplemented with 5% dialyzed FBS, 100 units penicillin/ml, 100 μg streptomycin/ml and 2mM L-glutamine/ml.

The time of initiation of chemical treatment was designated as day 0. Treatment was carried out by refeeding the treatment flasks with 5 ml F12FBS5-Hx/25 cm^2 flask for the non-activated study and 4 ml F12FBS5-Hx and 1 ml S9 reaction mixture/25 cm^2 flask for the S9-activated study, to which was added 50 μl dosing solution of test or control article

in solvent or solvent alone. Duplicate flasks of cells were exposed to at least five concentrations of the test article for 5 hours at 37±1°C. After the treatment period, all media were aspirated, the cells washed with Ca^{++}- and Mg^{++}-free Hanks' balanced salt solution (CMF-HBSS) and cultured in F12FBS5-Hx for an additional 18-24 hours at 37±1°C. At this time, the cells were subcultured to assess cytotoxicity and to initiate the phenotypic expression period.

Evaluation of Cytotoxicity

For evaluation of cytotoxicity, the replicates from each treatment condition were detached using trypsin and subcultured independently in F12FBS5-Hx, in triplicate, at a density of 100 cells/60 mm dish. After 7-10 days incubation, the colonies were rinsed with HBSS, fixed with methanol, stained with 10% aqueous Giemsa, counted and cloning efficiency determined.

Expression of the Mutant Phenotype

For expression of the mutant phenotype, the replicates from each treatment condition were trypsinized and subcultured independently in F12FBS5-Hx, in duplicate, at a density no greater than 10^6 cells/100 mm dish. Subculturing by trypsinizing at 2-3 day intervals was employed for the 7-9 day expression period. At the end of the expression period, selection for the mutant phenotype was performed.

Selection of the Mutant Phenotype

For selection of the TG-resistant phenotype, the replicates from each treatment condition were trypsinized and replated, in quintuplicate, at a density of $2x10^5$ cells/100 mm dish in F12FBS5-Hx containing 10 μM 6-thioguanine (TG, 2-amino-6-mercaptopurine). For cloning efficiency determinations at the time of selection, 100 cells/60 mm dish were plated in triplicate. After 7-10 days of incubation, the colonies were fixed, stained and counted for both cloning efficiency and mutant selection.

Evaluation of Test Results

The cytotoxic effects of each treatment condition were expressed relative to the solvent-treated control (relative cloning efficiency). The mutant frequency (MF) for each treatment condition was calculated by dividing the total number of mutant colonies by the number of cells selected (usually $2x10^6$ cells: 10 plates at $2x10^5$ cells/plate), corrected for the cloning efficiency of cells prior to mutant selection, and is expressed as TG-resistant mutants per 10^6 clonable cells. For experimental conditions in which no mutant colonies were observed, mutant frequencies were expressed as less than the frequency obtained with one mutant colony. Mutant frequencies generated from doses giving ≤10% relative survival are presented in the data but were not considered as valid data points.

Because spontaneous mutant frequencies are very low for the CHO/HGPRT assay,

calculation of mutagenic response in terms of fold increase in mutant frequency above the background rate does not provide a reliable indication of the significance of the observed response. The wide acceptable range in spontaneous mutant frequency also suggests the need to set a minimum mutant frequency for a response to be considered positive. Hsie *et al.* (1981) refer to a level of 50 mutants per 10^6 clonable cells. In this laboratory, the minimum level is set at >40 mutants per 10^6 clonable cells for a response to be considered positive.

The assay will be considered positive in the event of a dose-dependent increase in mutant frequencies with at least two consecutive doses showing mutant frequencies which are elevated above 40 mutants per 10^6 clonable cells. If a single point above 40 mutants per 10^6 clonable cells is observed at the highest dose, the assay will be considered suspect. If no culture exhibits a mutant frequency of >40 mutants per 10^6 clonable cells, the test article will be considered negative.

Criteria for a Valid Test

The cloning efficiency of the solvent control must be greater than 50%. The spontaneous mutant frequency in the solvent control must fall within the range of 0-25 mutants per 10^6 clonable cells. The positive control must induce a mutant frequency at least three times that of the solvent control and must exceed 40 mutants per 10^6 clonable cells.

Archives

Upon completion of the final report, all raw data and reports are maintained in Microbiological Associates, Inc.'s archives located at 9900 Blackwell Road, Rockville, Maryland 20850.

RESULTS AND DISCUSSION

Solubility Test

DMSO was determined to be the solvent of choice based on solubility of the test article and compatibility with the target cells. The test article was soluble in DMSO at a maximum concentration of approximately 250 mg base/ml.

Preliminary Toxicity Assay

The results of the preliminary toxicity assay are presented in Table 1. CHO cells were exposed to solvent alone and nine concentrations of test article ranging from 0.5 to 5000 μg base/ml in the absence and presence of S9 reaction mixture. The top dose of 5000 μg base/ml was delivered as a suspension from a stock of 500 mg base/ml DMSO into treatment medium. All other doses were delivered as solutions in DMSO into treatment medium. Test article precipitate was observed at dose levels of 1500 and 5000 μg base/ml in treatment medium. Treatment medium was cloudy but with no visible precipitate at concentrations of 50, 150 and 500 μg base/ml. Concentrations of

≤15 μg base/ml were soluble in treatment medium. The osmolality of the solvent control was 431 mOsm/kg. The osmolality of the top dose, 5000 μg base/ml, could not be determined because of heavy precipitate. However, the osmolality of 500 μg base/ml was 441 mOsm/kg. Cloning efficiency relative to the solvent controls (RCE) was 0% at 5 μg base/ml without activation and 1% at 15 μg base/ml with S9 activation. The 12% RCE at 500 μg base/ml is considered to be a spurious occurrence. Based on the results of the toxicity test, the doses chosen for the mutagenesis assay ranged from 1.0 to 4.0 μg base/ml and 2.5 to 10 μg base/ml for both the non-activated cultures and for the S9-activated cultures.

Mutagenesis Assay

The cytotoxic effects of the test article in the first trial of the mutagenesis assay (concurrent cytotoxicity) are presented in Table 2. After plating for concurrent cytotoxicity, all the cultures exhibited complete loss of cells during the expression period (data not shown) and were not cloned for mutant selection. The mutagenesis assay was repeated. Based on the concurrent cytotoxicity data from the first trial, the lower dose levels were essentially non-cytotoxic; therefore, a narrower dose range with focus on cytotoxic dose levels was selected for the second trial. The doses chosen for the second trial ranged from 2.0 to 4.0 μg base/ml for the non-activated cultures and from 6.0 to 10 μg base/ml for the S9-activated cultures. All concentrations of test article were delivered as solutions in DMSO into treatment medium and all concentrations remained soluble in treatment medium. The concurrent cytotoxicity data from the second trial of the mutagenesis assay are presented in Table 3. Mutagenicity data are presented in Tables 4 and 5. In the non-activated system, cultures treated with concentrations of 2.0, 2.5, 3.0, 3.5 and 4.0 μg base/ml were cloned. In the S9-activated system cultures treated with concentrations of 6.0, 7.0, 8.0, 9.0 and 10 μg base/ml were cloned. Relative cloning efficiency was 80% and 61% at the highest dose tested in the non-activated and S9-activated systems, respectively. None of the treated cultures exhibited mutant frequencies of greater than 40 mutants per 10^6 clonable cells.

CONCLUSION

All criteria for a valid study were met as described in the protocol. The results of the CHO/HGPRT Mutation Assay indicate that, under the conditions of this study, WR238605 Succinate did not cause a positive response in the non-activated and S9-activated systems and was concluded to be negative.

REFERENCES

Hsie, A.W., D.A. Casciano, D.B. Couch, B.F. Krahn, J.P. O'Neill, and B.L. Whitfield (1981) The use of Chinese hamster ovary cells to quantify specific locus mutation and to determine mutagenicity of chemicals. A report of the Gene-Tox Program, Mutation Research 86:193-214.

O'Neill, J.P., P.A. Brimer, R. Machanoff, G.P. Hirsch, and A.W. Hsie (1977) A quantitative assay of mutation induction at the hypoxanthine-guanine phosphoribosyl transferase locus in Chinese hamster ovary cells (CHO/HGPRT system): Development and definition of the system, Mutation Research 45:91-101.

TABLE 1

CHO/HGPRT MUTATION ASSAY

Preliminary Toxicity Assay Using WR238605 Succinate

-S9						+S9					
Treatment[1] (µg base/ml)	Colonies per dish 1	2	3	Cloning Efficiency[2]	Relative Cloning Efficiency[3] (%)	Treatment[1] (µg base/ml)	Colonies per dish 1	2	3	Cloning Efficiency[2]	Relative Cloning Efficiency[3] (%)
DMSO	92	110	110	1.04	100	DMSO	82	98	85	0.88	100
WR238605 Succinate:											
0.5	86	90	107	0.94	91	0.5	97	96	96	0.96	109
1.5	110	110	116	1.12	108	1.5	94	122	91	1.02	116
5.0	0	0	0	0.00	0	5.0	87	104	114	1.02	115
15	0	0	0	0.00	0	15	1	2	0	0.01	1
50	0	0	0	0.00	0	50	a	0	0	0.00	0
150	0	0	0	0.00	0	150	0	0	0	0.00	0
500	12	14	12	0.13	12	500	0	0	0	0.00	0
1500	0	0	0	0.00	0	1500	0	0	0	0.00	0
5000	0	0	0	0.00	0	5000	0	0	0	0.00	0

[1] Cells were exposed to the test article for 5 hours at 37±1°C.

[2] Cloning efficiency = $\frac{\text{total colonies counted}}{\text{number of dishes x 100 cells/dish}}$

[3] Relative cloning efficiency = $\frac{\text{cloning efficiency of treatment group}}{\text{cloning efficiency of solvent group}}$ X 100

[a] Plate lost due to contamination

TABLE 2

CHO/HGPRT MUTATION ASSAY

Concurrent Cytotoxicity Test Using WR238605 Succinate
First Trial

-S9							+S9						
Treatment[1] (µg base/ml)		Colonies per dish 1	2	3	Cloning Efficiency[2]	Relative Cloning Efficiency[3] (%)	Treatment[1] (µg base/ml)		Colonies per dish 1	2	3	Cloning Efficiency[2]	Relative Cloning Efficiency[3] (%)
DMSO	A	95	110	111			DMSO	A	139	130	134		
	B	105	102	143	1.11	100		B	82	76	77	1.06	100
EMS	A	138	135	147			B(a)P	A	30	33	42		
0.2 µl/ml	B	66	78	76	1.07	96	4 µg/ml	B	24	33	27	0.32	30
							B(a)P	A	29	30	32		
							5 µg/ml	B	35	31	19	0.29	28
WR238605 Succinate:													
1.0	A	104	99	a			2.5	A	161	160	161		
	B	83	101	86	0.95	85		B	96	107	81	1.28	120
1.5	A	105	114	131			5.0	A	89	91	99		
	B	104	102	97	1.09	98		B	93	93	102	0.95	89
2.0	A	104	107	112			6.0	A	76	115	95		
	B	83	90	109	1.01	91		B	90	96	95	0.95	89
2.5	A	116	108	98			7.0	A	99	92	106		
	B	100	88	74	0.97	88		B	78	72	74	0.87	82
3.0	A	44	40	41			8.0	A	37	50	48		
	B	81	86	71	0.61	55		B	46	40	49	0.45	42
4.0	A	4	9	8			10	A	19	13	7		
	B	21	21	26	0.15	13		B	6	6	14	0.11	10

[1] Cells were exposed to the test article for 5 hours at 37±1°C.

[2] Cloning efficiency = $\frac{\text{total colonies counted}}{\text{number of dishes x 100 cells/dish}}$

[3] Relative cloning efficiency = $\frac{\text{cloning efficiency of treatment group}}{\text{cloning efficiency of solvent group}}$ X 100

EMS Ethyl methanesulfonate
B(a)P Benzo(α)pyrene
[a] Plate lost due to contamination

TABLE 3

CHO/HGPRT MUTATION ASSAY

Concurrent Cytotoxicity Test Using WR238605 Succinate
Second Trial

-S9							+S9						
Treatment[1] (µg base/ml)		Colonies per dish 1	2	3	Cloning Efficiency[2]	Relative Cloning Efficiency[3] (%)	Treatment[1] (µg base/ml)		Colonies per dish 1	2	3	Cloning Efficiency[2]	Relative Cloning Efficiency[3] (%)
DMSO	A	71	78	75			DMSO	A	76	70	65		
	B	78	63	72	0.73	100		B	101	94	112	0.86	100
EMS	A	67	62	61			B(a)P	A	9	11	6		
0.2 µl/ml	B	32	38	47	0.51	70	4 µg/ml	B	16	20	22	0.14	16
							B(a)P	A	21	21	16		
							5 µg/ml	B	16	9	8	0.15	18
WR238605 Succinate:													
2.0	A	80	62	63			6.0	A	53	58	69		
	B	86	105	82	0.80	109		B	87	96	98	0.77	89
2.5	A	83	81	65			7.0	A	66	52	68		
	B	67	74	a	0.74	102		B	67	76	65	0.66	76
3.0	A	46	31	37			8.0	A	49	58	62		
	B	67	58	66	0.51	70		B	37	34	35	0.46	53
3.5	A	62	a	56			9.0	A	10	12	10		
	B	49	48	a	0.54	74		B	26	33	23	0.19	22
4.0	A	76	75	84			10	A	17	10	3		
	B	31	42	40	0.58	80		B	96	84	107	0.53	61

[1] Cells were exposed to the test article for 5 hours at 37±1°C.

[2] $\text{Cloning efficiency} = \dfrac{\text{total colonies counted}}{\text{number of dishes x 100 cells/dish}}$

[3] $\text{Relative cloning efficiency} = \dfrac{\text{cloning efficiency of treatment group}}{\text{cloning efficiency of solvent group}} \text{ X 100}$

EMS Ethyl methanesulfonate
B(a)P Benzo(α)pyrene
[a] Plate lost due to contamination

TABLE 4

CHO/HGPRT MUTATION ASSAY

Non-activated (-S9) Study Using WR238605 Succinate
Second Trial

Treatment[1] (µg base/ml)		Cloning Efficiency at Selection: Colonies per Dish 1	2	3	Total Colonies	Cloning Efficiency[2]	Mutant Colonies/Selection Dish 1	2	3	4	5	Total Mutant Colonies	Mutants/10^6 Clonable Cells[3]
DMSO	A	102	104	100			0	0	0	0	0		
	B	55	78	67	506	0.84	0	0	0	0	a	0	<0.7[4]
EMS	A	81	101	107			41	42	45	42	52		
0.2 µl/ml	B	83	97	87	556	0.93	45	29	23	39	48	406	219.1
WR238605 Succinate:													
2.0	A	92	82	71			0	1	0	0	0		
	B	103	105	123	576	0.96	1	0	0	0	2	4	2.1
2.5	A	102	103	106			0	0	0	0	0		
	B	98	105	125	639	1.07	6	0	0	0	0	6	2.8
3.0	A	80	80	86			0	0	0	0	0		
	B	95	100	109	550	0.92	0	a	a	0	0	0	<0.7[4]
3.5	A	84	91	101			0	1	1	1	1		
	B	119	127	113	635	1.06	15	a	6	9	11	45	23.6
4.0	A	71	108	69			0	0	0	0	0		
	B	108	102	109	567	0.95	1	1	0	1	a	3	1.8

[1] Cells were exposed to the test article for 5 hours at 37±1°C.

[2] Cloning efficiency = $\dfrac{\text{total colonies counted}}{\text{number of dishes x 100 cells/dish}}$

[3] Mutants/10^6 clonable cells = $\dfrac{\text{total mutant colonies}}{\text{number selection dishes X cloning efficiency X 2 x } 10^5 \text{ cells}} \times 10^6$

[4] Calculated on the basis of <1 mutant colonies observed in the total number of dishes prepared.

EMS Ethyl methanesulfonate.

[a] Plate lost due to contamination

TABLE 5

CHO/HGPRT MUTATION ASSAY

Activated (+S9) Study Using WR238605 Succinate
Second Trial

Treatment[1] (µg base/ml)		Cloning Efficiency at Selection: Colonies per Dish 1	2	3	Total Colonies	Cloning Efficiency[2]	Mutant Colonies/Selection Dish 1	2	3	4	5	Total Mutant Colonies	Mutants/10^6 Clonable Cells[3]
DMSO	A	86	89	102			0	0	a	0	1		
	B	99	98	83	557	0.93	0	0	0	0	0	1	0.6
B(a)P	A	92	62	117			a	44	37	28	a		
4 µg/ml	B	80	75	78	504	0.84	23	25	15	22	17	211	157.0
B(a)P	A	94	83	86			a	a	a	25	21		
5 µg/ml	B	89	74	71	497	0.83	23	a	21	19	23	132	132.8
WR238605 Succinate:													
6.0	A	74	76	78			0	0	0	1	0		
	B	81	82	95	486	0.81	a	0	0	0	0	1	0.7
7.0	A	101	121	98			0	0	1	0	0		
	B	81	104	81	586	0.98	3	1	a	0	3	8	4.6
8.0	A	95	98	70			0	0	0	0	0		
	B	75	83	83	504	0.84	1	0	0	0	0	1	0.6
9.0	A	88	102	88			a	a	0	0	0		
	B	90	76	87	531	0.89	4	1	0	3	2	10	7.1
10	A	94	77	a			0	0	0	0	0		
	B	84	104	98	457	0.91	14	15	16	a	18	63	38.3

[1] Cells were exposed to the test article for 5 hours at 37±1°C.

[2] Cloning efficiency = $\frac{\text{total colonies counted}}{\text{number of dishes x 100 cells/dish}}$

[3] Mutants/10^6 clonable cells = $\frac{\text{total mutant colonies}}{\text{number selection dishes X cloning efficiency X 2 x } 10^5 \text{ cells}} \times 10^6$

B(a)P Benzo(a)pyrene.

[a] Plate lost due to contamination

APPENDIX I

Historical Control Data

CHO/HGPRT Assay Historical Control Data

1992 - 1994

	Non-activated			S9-Activated		
	Untreated Control	Solvent Control	0.2μl/ml EMS	Untreated Control	Solvent Control	4μg/ml B(a)P
Mean MF	6.1	7.6	224.8	6.3	8.3	116.4
SD	4.2	5.2	54.6	5.1	6.7	50.9
Maximum	22.4	19.9	387.5	22.0	24.2	233.7
Minimum	0.5	0.4	121.1	0.4	0.4	48.2

Solvent controls (culture medium, distilled water, saline, DMSO, ethanol, acetone or vehicle supplied by Sponsor)

EMS Ethyl methanesulfonate
B(a)P Benzo(a)pyrene
MF Mutant frequency per 10^6 clonable cells
SD Standard deviation

APPENDIX II

Study Protocol

Received by RA/QA 2-9-95

MA Study Number: G94BE97.782

CHO/HGPRT MUTATION ASSAY

1.0 PURPOSE

The purpose of this study is to assess the mutagenic potential of a test article based on quantitation of forward mutations at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus of Chinese hamster ovary (CHO) cells.

2.0 SPONSOR

2.1 Name: **Toxicology Research Laboratory**

2.2 Address: University of Illinois at Chicago
Department of Pharmacology
1940 West Taylor Street
Chicago, IL 60612-7353

2.3 Representative: Barry S. Levine, D.Sc., D.A.B.T.

2.4 Sponsor Project #: UIC-10 This study will be conducted by Microbiological Associates, Inc. and is being sponsored by the Toxicology Research Laboratroy, UIC under a contract from U.S. Army Medical Research Acquisition Activity (No. DAMD 17-92-C-2001). Dr. George Schieferstein, Contracting Officer's Representative.

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1 Test Article: **WR238605 Succinate** (base mole fraction = 0.8)

Storage: 2 - 8°C
Ambient humidity conditions
Protect from light

3.2 Controls: Solvent: Test article vehicle
Positive: Ethyl methanesulfonate (EMS)
Benzo(a)pyrene (BaP)

3.3 Determination of Strength, Purity, etc.

The Sponsor will be directly responsible for determination and documentation of the analytical purity and composition of the test article and the stability and strength of the dosing solutions.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1 Name: Genetic Toxicology Division
Microbiological Associates, Inc.

4.2 Address: 9900 Blackwell Road
Rockville, MD 20850

4.3 Study Director: Richard H. C. San, Ph.D.

5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date: 2/15/95

5.2 Proposed Experimental Completion Date: 4/6/95

5.3 Proposed Report Date: 4/14/95

6.0 TEST SYSTEM

The CHO-K1-BH4 cell line is a proline auxotroph with a modal chromosome number of 20, a population doubling time of 12-14 hours, and a cloning efficiency of usually greater than 80% (1). This subclone (D1) was derived by Dr. Abraham Hsie, Oak Ridge National Laboratories, Oak Ridge, TN. CHO cells were cleansed in medium supplemented with HAT (hypoxanthine, aminopterin and thymidine) then frozen. Cells used in the mutation assay will not exceed four subpassages from frozen stock. Each freeze lot of cells has been tested and found to be free of mycoplasma contamination.

The CHO/HGPRT assay was designed to select for mutant cells which have become resistant to such purine analogues as 6-thioguanine (TG) and 8-azaguanine as a result of mutation at the X-chromosome-linked HGPRT locus (1-4). This system has been demonstrated to be sensitive to the mutagenic action of a variety of chemicals (2).

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The assay will be performed by exposing CHO cells for 5 hours to five concentrations of test article as well as positive and the solvent controls in the presence and absence of an exogenous source of metabolic activation. After a seven to nine day expression period, the treated cells will be cultured in the presence of 10 μM TG for selection of mutant colonies. The mutagenic potential of a test article will be determined by its ability to induce a dose-related increase in the number of TG-resistant mutant colonies when compared to the solvent control.

7.1 Selection of solvent

Unless the Sponsor has indicated the test article solvent, a solubility determination will be conducted to measure the maximum soluble concentration in a variety of solvents. Solvents compatible with this test system, in order of preference, include, but are not limited to, culture medium or distilled water (CAS 7732-18-5), dimethylsulfoxide (CAS 67-68-5), ethanol (CAS 64-17-5) and acetone (CAS 67-64-1). The solvent of choice will be that solvent, selected in order of preference, that permits preparation of the highest soluble stock concentration, up to a maximum of 500 mg/ml.

7.2 Dose Selection

The optimal dose levels for the mutation assay will be selected following a preliminary toxicity test based upon colony-forming efficiency. Approximately 5 x 10^5 CHO cells will be seeded into 25 cm^2 flasks and incubated at 37 ± 1°C in a humidified atmosphere of $5\pm1\%$ CO_2 in air. Eighteen to 24 hours later, cells will be exposed to solvent alone and to no less than nine concentrations of test article, the highest concentration being the lowest insoluble dose in treatment medium not to exceed 5000 μg/ml. The pH of the treatment medium will be adjusted, if necessary, to maintain a neutral pH in the treatment medium. The osmolality of the highest soluble treatment condition will also be measured. Exposure will be for 5 hours at 37 ± 1°C in a humidified atmosphere of $5\pm1\%$ CO_2 in air in the presence and absence of S-9 activation. Eighteen to 24 hours after removal of treatment medium, the treated cells will be trypsinized and reseeded at a density of 100 cells/60 mm dish. After 7-10 days incubation at 37 ± 1°C in a humidified atmosphere of $5\pm1\%$ CO_2 in air, colonies will be fixed with 95% methanol, stained with 10% aqueous Giemsa, and counted. The cell survival of the test article-treated groups will be expressed relative to the solvent control (relative cloning efficiency).

Whenever possible, the high dose will be selected to give a cell survival of 10-30%. Four lower doses will be selected, at least one of which will be non-toxic. If the desired toxicity is not achieved in the preliminary toxicity test at the maximum concentration allowed by protocol, the Sponsor will be contacted prior to dose selection.

7.3 Frequency and Route of Administration

Cell cultures will be treated for 5 hours by way of a vehicle compatible with the system, both in the presence and absence of metabolic activation. This technique of administration has been demonstrated to be effective in the detection of chemical mutagens in this system.

7.4 Exogenous Metabolic Activation

Aroclor 1254-induced rat liver S-9 will be used as the metabolic activation

system. The source of S-9 will be adult male Sprague-Dawley rats induced by a single injection of Aroclor 1254 at a dose level of 500 mg/kg body weight five days prior to sacrifice. The S-9 will be batch prepared and stored frozen approximately -70°C until used.

Immediately prior to use, the S-9 will be thawed and mixed with a cofactor pool to contain 100 μl S-9/ml reaction mixture of approximately 4 mM NADP, 5 mM glucose-6-phosphate, 10 mM $MgCl_2$, 30 mM KCl, 10 mM $CaCl_2$, and 50 mM sodium phosphate buffer, pH 8.0 (3). The S-9 reaction mixture will be stored on ice until used.

7.5 Controls

7.5.1 Solvent control

The solvent for the test article will be used as the solvent control.

7.5.2 Positive control

Ethyl methanesulfonate (EMS) will be used at one concentration within the range of 0.1 to 0.4 μl/ml as the positive control for the non-activated study. Benzo(a)pyrene (BaP) will be used at one or two concentrations within the range of 3 to 6 μg/ml as the positive control for the S-9 activated study.

7.6 Preparation of Target Cells

Exponentially growing CHO-K1-BH4 cells will be seeded in F12 medium, with or without hypoxanthine, supplemented with 5% dialyzed serum (F12FBS5 or F12FBS5-Hx) at a density of 5 x 10^5 cells/25 cm^2 surface area and will be incubated at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air for 18-24 hours.

7.7 Identification of the Test System

Using a permanent marking pen, the treatment flasks will be identified by the study number and a code system to designate the treatment condition and test phase.

7.8 Treatment of Target Cells

The time of initiation of chemical treatment will be designated as day 0. Cells will be exposed, in duplicate cultures, to five concentrations of test article for 5 hours at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air. For each 25 cm^2 of surface area treated, the treatment medium will consist of 5 ml F12FBS5 or F12FBS5-Hx and 50 μl of control or test article diluted to the appropriate concentration in solvent for the non-activated study, or 4 ml F12FBS5 or F12FBS5-Hx, 1 ml S-9 reaction mixture, and 50 μl of

control or test article diluted to the appropriate concentration in solvent for the activated study. After the treatment period, all media will be aspirated, the cells washed with Hank's Balanced Salt Solution (CMF-HBSS) and cultured in F12FBS5 or F12FBS5-Hx at 37±1°C in a humidified atmosphere of 5±1% CO_2 in air. After 18-24 hours incubation, the cells will be subcultured to assess cytotoxicity and to continue the phenotypic expression period.

7.9 Estimation of Toxicity

For evaluation of cytotoxicity, the replicate cultures from each treatment condition will be subcultured independently in F12FBS5 or F12FBS5-Hx, in triplicate, at a density of 100 cells/60 mm dish. After 7-10 days incubation at 37±1°C in 5±1% CO_2 in air, colonies will be fixed with 95% methanol, stained with 10% aqueous Giemsa, and counted. Cytotoxicity will be expressed relative to the solvent-treated control cultures.

7.10 Expression of the Mutant Phenotype

For expression of the mutant phenotype, the replicates from each treatment condition will be subcultured independently in F12FBS5 or F12FBS5-Hx, at a density of no greater than 10^6 cells/100 mm dish. Subculture as above at 2-3 day intervals will be performed for the 7-9 day expression period. At this time, selection for the mutant phenotype will be performed.

7.11 Selection of the Mutant Phenotype

For selection of the TG-resistant phenotype, cells from each treatment condition will be plated into a maximum of five dishes at a density of 2 x 10^5 cells/100 mm dish in F12FBS5-Hx containing 10 μM TG. For cloning efficiency at the time of selection, 100 cells/60 mm dish will be plated in triplicate in medium free of TG. After 7-10 days of incubation, the colonies will be fixed, stained and counted for both cloning efficiency at selection and mutant selection.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

The cloning efficiency of the solvent control must be greater than 50%. The spontaneous mutant frequency in the solvent control must fall within the range of 0-25 mutants per 10^6 clonable cells.

The positive control must induce a mutant frequency at least three times that of the solvent control and must exceed 40 mutants per 10^6 clonable cells.

9.0 EVALUATION OF TEST RESULTS

The cytotoxic effects of each treatment condition are expressed relative to the solvent-treated control (relative cloning efficiency). The mutant frequency (MF) for

each treatment condition is calculated by dividing the total number of mutant colonies by the number of cells selected, corrected for the cloning efficiency of cells prior to mutant selection, and is expressed as TG-resistant mutants per 10^6 clonable cells. For experimental conditions in which no mutant colonies are observed, mutant frequencies will be expressed as less than the frequency obtained with one mutant colony. Mutant frequencies generated from doses giving $\leq 10\%$ relative survival are not considered as valid data points and will not be included in the data analysis.

Spontaneous mutant frequencies in this assay range from 0 to 25 mutants per 10^6 clonable cells. As a result, calculation of mutagenic response in terms of fold increase in mutant frequency above the background rate does not provide a reliable indication of the significance of the observed response. The wide acceptable range in spontaneous mutant frequency also suggests the need to set a minimum mutant frequency for a response to be considered positive. Hsie *et al* (2) refer to a level of 50 mutants per 10^6 clonable cells. In this laboratory, a more conservative approach is used which sets the minimum significant level at >40 mutants per 10^6 clonable cells.

All conclusions will be based on sound scientific judgement; however, the following will be used as a guide to interpretation of the data. The test article will be considered to induce a positive response if there is a concentration-related increase in mutant frequencies with at least two consecutive doses showing mutant frequencies of > 40 mutants per 10^6 clonable cells. If a single point above 40 mutants per 10^6 clonable cells is observed at the highest dose, the assay will be considered suspect. If no culture exhibits a mutant frequency of > 40 mutants per 10^6 clonable cells, the test article will be considered negative.

10.0 REPORT

A report of the results of this study will be prepared by the Testing Laboratory and will accurately describe all methods used for generation and analysis of data.

Results presented will include, but not be limited to:

- cell type used, number of cultures, methods for maintenance of cell cultures
- rationale for selection of concentrations and number of cultures
- test conditions: composition of media, CO_2 concentration, concentration of test substance, vehicle, incubation temperature, incubation time, duration of treatment, cell density during treatment, type of metabolic activation system, positive and negative controls, length of expression period, selective agent
- method used to enumerate numbers of viable and mutant cells
- dose-response relationship, if applicable
- positive and solvent control historical data

11.0 RECORDS AND ARCHIVES

Upon completion of the final report, all raw data and reports will be maintained by the Regulatory Affairs Unit of Microbiological Associates, Inc. in accordance with the relevant Good Laboratory Practice Regulations.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol was written according to the OECD Guideline 476 (Genetic Toxicology: *In Vitro* Mammalian Cell Gene Mutation Tests), April, 1984; and the EPA Health Effects Testing Guidelines, Subpart 798.5300 (Detection of Gene Mutations in Somatic Cells in Culture) Fed. Register, vol. 50, September, 1985 with revisions Fed. Register, vol. 52, May, 1987. A confirmatory assay will be required for full OECD and EPA guideline compliance.

This study will be performed in compliance with the provisions of the Good Laboratory Practice Regulations for Nonclinical Laboratory Studies.

Will this study be submitted to a regulatory agency? _yes_

If so, to which agency or agencies? _US FDA_

Unless arrangements are made to the contrary, the Test Article will be returned to the Sponsor at the conclusion of the study. In addition, does the Sponsor request that samples of the Test Article dosing solutions be returned? _yes_
If so, indicate which doses and volume will be required.

______high only;

______high and low only;

___X__all;

volume required ___10 ml (if possible)___.

The dosing solutions will be frozen and sent to UIC for analysis.

13.0 REFERENCES

1. Li, A. P., J. H. Carver, W. N. Choy, A. W. Hsie, R. S. Gupta, K. S. Loveday, J. P. O'Neill, J. C. Riddle, L. F. Stankowski, and L. L. Yang. 1987. A guide for the performance of Chinese hamster ovary cell/hypoxanthine-guanine phosphoribosyl transferase gene mutation assay. Mutation Res. 189: 135-141.

2. Hsie, A. W., D. A. Casciano, D. B. Couch, B. F. Krahn, J. P. O'Neill, and B. L. Whitfield. 1981. The use of Chinese hamster ovary cells to quantify specific locus mutation and to determine mutagenicity of chemicals. A report of the Gen-Tox

Program. Mutation Res. 86: 193-214.

3. Machanoff, R., J. P. O'Neill, and A. W. Hsie. 1981. Quantitative analysis of cytotoxicity and mutagenicity of benzo(a)pyrene in mammalian cells (CHO/HGPRT). Chem. Biol. Interactions. 34: 1-10.

4. O'Neill, J. P., P. A. Brimer, R. Machanoff, J. P. Hirsch, and A. W. Hsie. 1977. a quantitative assay of mutation induction at the hypoxanthine-guanine phosphoribosyl transferase locus in Chinese hamster ovary cells (CHO/HGPRT system): Development and definition of the system. Mutation Res. 45: 91-101.

14.0 APPROVAL

Barry S. Levine 1/5/95

Barry S. Levine, D.Sc., D.A.B.T. (Sponsor Representative) Date

1/24/95

Dr. George Schieferstein (US Army Contracting Officer's Representative) Date

2/9/95

MA Study Director Date

APPENDIX 4

ANALYSIS OF DOSING SOLUTIONS

Analysis of Mutagenicity Dosing Solutions of WR238605 for Microbiological Associates Inc. Study Numbers G94BE97.330, G94BE97.702, and G94BE97.782

Analyst: Thomas Tolhurst

Study Site: Drug Disposition Research Laboratory
College of Pharmacy
University of Illinois at Chicago
Chicago, Illinois 60612

Sponsor: Toxicology Research Laboratory
University of Illinois at Chicago
Chicago, Illinois 60612

Report Prepared by: Thomas Tolhurst
T. Tolhurst

Report Prepared: January 15, 1996

Approved: January 18, 1996
Eugene F. Woods E. Woods
Drug Disposition Research Laboratory

Analytical Method

Introduction

All dosing solution samples for the quantitation of WR238605 Succinate were submitted frozen by the Toxicology Research Laboratory (TRL). The concentrations of WR238605 (free base) were determined by high performance liquid chromatography (HPLC) using a C18 column for separation and UV detection at 268 nm. Standard curves were analyzed at the beginning and at the end of each assay run.

Reagents

Subject sample to be analyzed for WR238605 (free base) were submitted by TRL. HPLC grade methanol, 85% o-phosphoric acid, sodium acetate and Certified A.C.S. dimethyl sulfoxide were purchased from Fisher Scientific. HPLC quality water was supplied through a Millipore®, MILLI -Q® Reagent water system which was fed with distilled water.

Standards

All WR238605 concentrations reflect free base values. A stock solution containing 0.8 mg base/ml WR238605 was prepared by weighing 100 mg of WR238605 succinate (mole fraction 0.8) into a 100 ml volumetric flask. The contents were dissolved in and the volume brought to mark with DMSO. A 1.0 ml aliquot was transferred to another 100 ml volumetric flask. The volume was brought to mark with DMSO resulting in a WR238605 base concentration of 80 µg/ml. Calibration standard solutions were prepared in DMSO using the 80 µg base/ml WR238605 solution as follows:

Volume Transferred (ml)	Flask Volume (ml)	Final Conc. (µg base/ml)
1.0	10	8
2.0	10	16
4.0	10	32
6.0	10	48
8.0	10	64

Standard curves were prepared as described above and were linear over the range of WR238605 assayed (8.0 - 80 µg base/ml). A representative curve is shown in Figure 1.

Controls

Low control stock solutions were prepared by weighing 25 mg of WR238605 Succinate into a 25 ml volumetric flask. The contents were dissolved in and diluted to volume with DMSO. Working low control solutions were prepared by transferring 1.0 ml of the stock solution to a 25 ml volumetric flask and diluting to mark with DMSO. The working low control solutions were then analyzed for WR238605 (free base).

High control stock solutions were prepared by weighing 75 mg of WR238605 Succinate in a 25 ml volumetric flask. The contents were dissolved in and diluted to mark with DMSO. Working high control solutions were prepared by diluting the stock solution 1:62.5 in DMSO (1:25 dilutions followed by a 1:2.5 dilution). The working high control solutions were then analyzed for WR238605 (free base).

Samples

Subject samples had been prepared by Microbiological Associates Inc. using DMSO as the vehicle. All samples were stored frozen until analyzed for WR238605 free base. When needed, samples were diluted in mobile phase allowing the final concentration of WR238605 free base to fall within the range of the standard curve (8 to 80 μg/ml). When it was necessary to dilute the subject samples for analysis, standard curves and controls were also prepared in DMSO corresponding to the dilution required for the subject sample concentration to fall within the range of the standard curve.

HPLC System

Solvent Delivery System:	Waters 510 Pump
Injector:	Rheodyne 7125, with 50 µl sample loop
Analytical Column:	Bondclone, C18, 10µ, 250 x 4.6 mm (Phenomenx)
Detector:	Kratos, Spectroflow 77 UV Detctor
Integrator:	Perkin-Elmer LCI-100 Intergrator
Mobile Phase:	750 ml of methanol: 250ml of deionized water to which was added 9.0 ml of 85% o-phosphoric acid and 6.9 g of sodium acetate.

Calculations

A standard curve was run at the beginning and the end of each day's assay. Final concentrations for controls and samples were determined using a composite standard curve. The composite curve was determined by linear least squared regression analysis of the peak heights for WR238605 as a function of concentration. WR238605 concentrations (mg base/ml) for controls and samples were determined using the following equation:

$$\text{WR238605 Conc. (mg/ml)} = (Y-B)/M \times (\text{d.f.}/1000\ \mu\text{g/mg})$$

Y = peak height

B = Y-intercept from composite standard curve

M = slope from composite standard curve

d.f. = dilution factor

Results

Results of dosing formulations analysis used in the assessment of mutagenicity in Study Numbers G94BE97.330, G94BE97.702 and G94BE97.782 conducted by Microbiological Associates, Inc. are presented in Tables 1, 2, and 3 respectively.

Approximately 90% of the samples (24/27) were within 10% of target concentration. All dosing solutions used in Study Nos. G94BE97.782 and G94BE97.330 and the 6 highest concentrations of dosing solutions (out of a total of 9 solutions) used in Study No. G94BE97.702 were within target.

FIGURE 1

WR238605 Standard Curve in DMSO

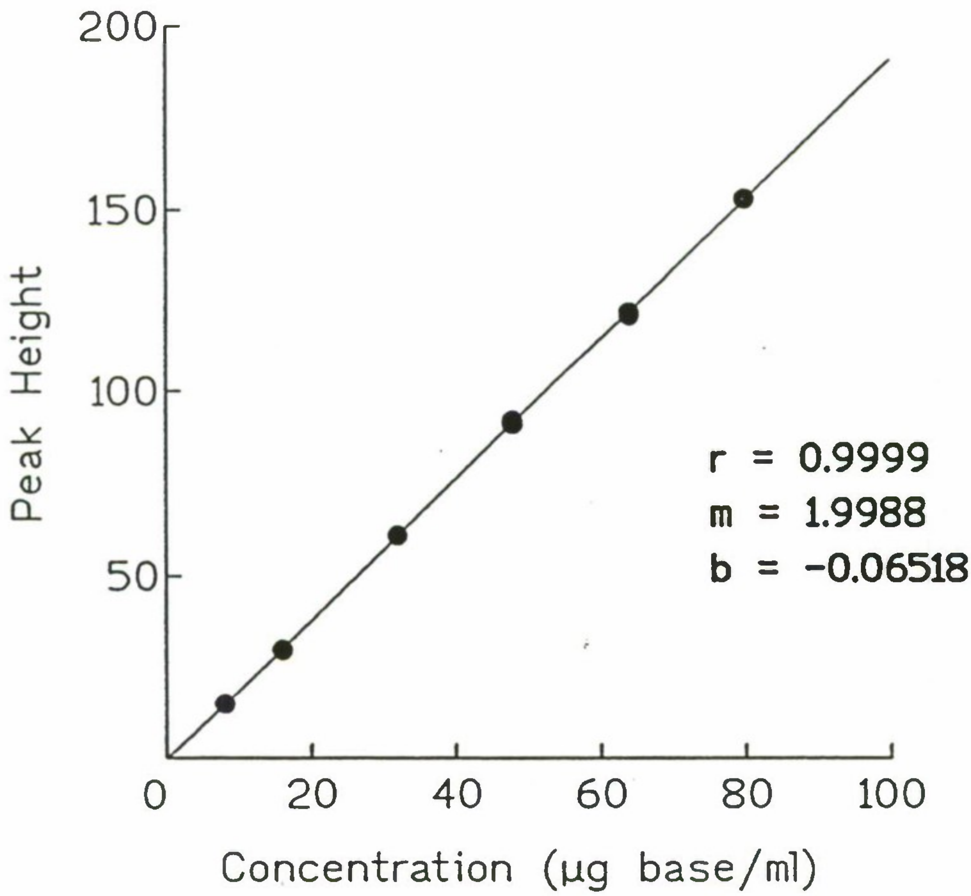
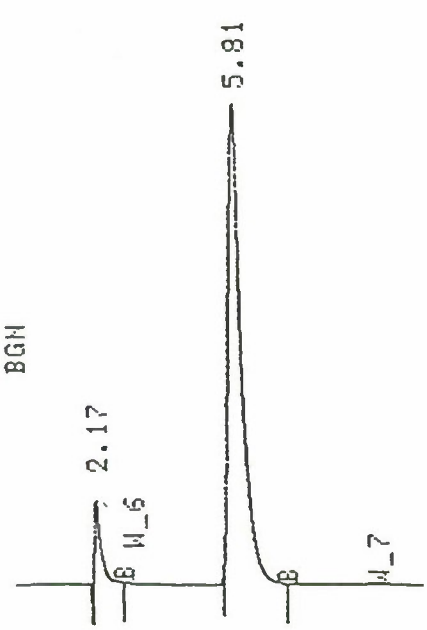
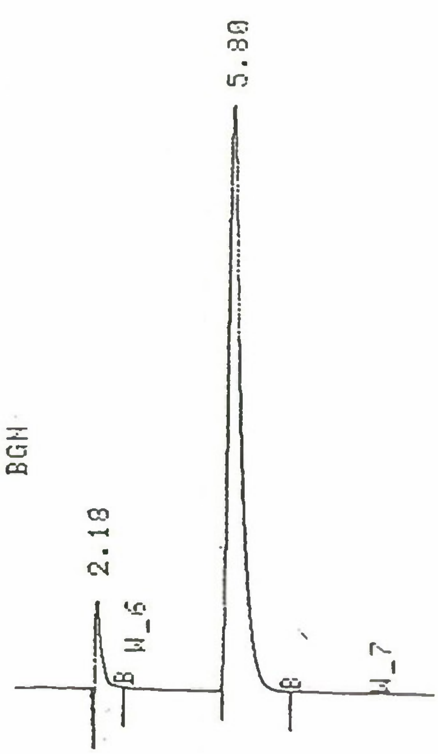


Figure 2

WR238605 Representative Chromatograms



Low Control



High Control

Table 1

Results of WR238605 Mutagenicity Dosing Solutions Analysis for Study No. G94BE97.330

Target Concentration (mg base/ml)	Mean Measured Conc. (mg base/ml)	% Target
0.18	0.177 ± 0.0002	98.3
0.22	0.220 ± 0.0001	100.0
0.28	0.274 ± 0.0004	97.9
0.35	0.348 ± 0.0024	99.4
0.56	0.568 ± 0.0025	101.4
0.7	0.693 ± 0.0103	99.0
0.88	0.913 ± 0.0111	103.8
1.1	1.131 ± 0.0027	102.8

Table 2

Results of WR238605 Mutagenicity Dosing Solutions Analysis for Study No. G94BE97.702

Target Concentration (μg base/ml)	Mean Measured Conc. (μg base/ml)	% Target
50	22.208 $\pm$ 0.664	44.4
75	36.460 $\pm$ 1.0307	48.6
100	48.810 $\pm$ 0.288	48.8
150	146.377 $\pm$ 0.293	97.6
200	199.378 $\pm$ 0.781	99.7
250	251.193 $\pm$ 0.575	100.5
300	300.674 $\pm$ 1.610	100.2
400	403.431 $\pm$ 0.138	100.9
500	499.226 $\pm$ 0.315	99.8

Table 3

Results of WR238605 Mutagenicity Dosing Solutions Analysis for Study No. G94BE97.782

Target Concentration (μg base/ml)	Mean Measured Conc. (μg base/ml)	% Target
200	211.308 $\pm$ 4.507	105.7
250	254.010 $\pm$ 10.278	101.6
300	312.209 $\pm$ 7.872	104.1
350	358.796 $\pm$ 6.461	102.5
400	411.396 $\pm$ 7.473	102.8
600	610.923 $\pm$ 6.805	101.8
700	711.795 $\pm$ 1.389	101.7
800	835.500 $\pm$ 5.700	104.4
900	945.500 $\pm$ 1.000	105.1
1000	1016.400 $\pm$ 10.400	101.6

QUALITY ASSURANCE STATEMENT

STUDY TITLE: ANALYSIS OF MUTAGENICITY DOSING SOLUTIONS OF WR238605 FOR MICROBIOLOGICAL ASSOCIATES, INC. FOR STUDY NUMBERS G94BE97.330, G94BE97.702, AND G94BE97.782.

The following are the inspection date and report date of QA inspections of this study.

INSPECT ON 1/16/96, TO STUDY DIR 1/17/96, TO MGMT 1/19/96
PHASES: RAW DATA AND FINAL REPORT FROM THE DRUG DISPOSITION RESEARCH LABORATORY

Ronald Schlesinger 1/19/96

QUALITY ASSURANCE DATE

APPENDIX 5

KEY TO DOSING SOLUTIONS TESTED FOR MUTAGENICITY

G94BE97.330: clarification of dose levels used in the study

Dosing Solution Concentration (mg base/ml)	Final Concentration -S9 (µg base/ml)	Final Concentration +S9 (µg base/ml)
B1 Study:		
0.18	1.8	NT
0.22	2.2	NT
0.28	2.8	NT
0.35	3.5	NT
0.43	NS	NT
0.54	NS	NT
0.45	NT	NS
0.56	NT	5.6
0.7	NT	7
0.88	NT	8.8
1.1	NT	11
1.4	NT	NS
1.8	NT	NS

NT = not tested
NS = not scored

Dose Levels for MA Study Number G94BE97.702
(Sponsor Study Number UIC-10)

Dosing Solution Concentration (μg base/ml)	Final Concentration in Treatment Medium (μg base/ml)	
	Without S9	With S9
12.5	Not Tested	Tested (not cloned)
25	Not Tested	Tested (not cloned)
50	Tested (not cloned)	0.5
75	Not Tested	0.75
100	1.0	1.0
150	1.5	Not Tested
200	2.0	Not Tested
250	2.5	2.5
300	3.0	Not Tested
400	4.0	Not Tested
500	Tested (not cloned)	5.0
1000	Not Tested	Tested (not cloned)

Dose Levels for MA Study Number G94BE97.782
(Sponsor Study Number UIC-10)

Dosing Solution Concentration (μg base/ml)	Final Concentration in Treatment Medium (μg base/ml)	
	Without S9	With S9
200	2.0	Not Tested
250	2.5	Not Tested
300	3.0	Not Tested
350	3.5	Not Tested
400	4.0	Not Tested
600	Not Tested	6.0
700	Not Tested	7.0
800	Not Tested	8.0
900	Not Tested	9.0
1000	Not Tested	10